



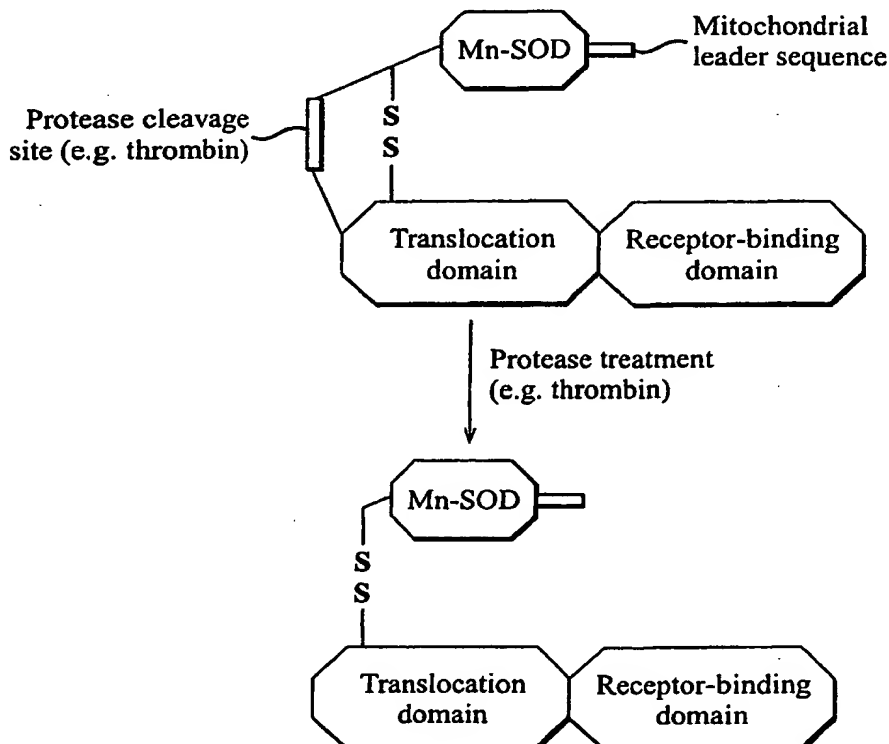
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(54) Title: DELIVERY OF SUPEROXIDE DISMUTASE TO NEURONAL CELLS

(57) Abstract

A composition for delivery of superoxide dismutase to neuronal cells comprise a superoxide dismutase linked by a linker to a neuronal cell targeting component, which component comprises a first domain that binds to a neuronal cell and a second domain that translocates the superoxide dismutase into the neuronal cell. After translocation, the linker is cleaved to release superoxide dismutase from the neuronal cell targeting domain. Also described is use of the composition for treatment of oxidative damage to neuronal cells and further targeting of the composition using human mitochondrial leader sequences. A hybrid polypeptide is described that contains a bacterial superoxide dismutase plus a sequence that targets a human mitochondria.



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DELIVERY OF SUPEROXIDE DISMUTASE TO NEURONAL CELLS

The present invention relates to compositions and methods for delivery of superoxide dismutase (SOD) to neuronal cells, and in particular for delivery of SOD to mitochondria in those cells. The present invention also provides methods of making the constructs.

A number of nervous system disorders would benefit considerably from rapid intervention with several therapeutic agents. Examples of such disorders are global, focal or spinal cord ischaemia induced by stroke or injury. Neurones injured by trauma or ischaemia produce elevated levels of transmitter substances which result in high levels of reactive oxygen species. These reactive oxygen species, in high concentrations are toxic to both the neurones and the surrounding cells which potentiates and amplifies the damage process. Rapid therapeutic intervention with agents to reduce oxidative stress in cases of neuronal injury caused by stroke or trauma could therefore significantly limit this secondary damage process.

One such potential therapeutic agent is superoxide dismutase which neutralises the harmful effects of the superoxide radicals by converting them to hydrogen peroxide and oxygen.

Francis and co-workers demonstrated that post-ischaemic infusion of Copper/Zinc superoxide dismutase (Cu/Zn-SOD) reduces cerebral infarction following ischaemia/reperfusion in rats (Experimental Neurology (1997) 146, 435-443) through the reduction of damaging free-radical oxygen. Lim et al. have shown that administration of Cu/Zn-SOD attenuates the level of reperfusion injury following spinal cord ischaemia in dogs (Ann. Thorac. Surg. (1986) 42, 282-286). Cuevas et al. have similarly demonstrated protective effects of SOD, both on neurological recovery and spinal infarction, in ischaemic reperfusion injury of the rabbit spinal cord (Acta Anat. (1990) 137, 303-310). A major problem in the use of such therapies is the maintenance of useful concentration of the active agent at the site

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of trauma. Enzymes such as Cu/Zn-SOD are rapidly cleared from the systemic circulation; in the case of the latter enzyme the $t_{1/2}$ in rat is 4-8 minutes. A number of strategies have been employed to overcome these difficulties. Matsumiya et al. (Stroke (1991) 22, 1193-1200) conjugated Cu/Zn-SOD to polyethylene glycol to increase its half-life in the blood. Francis and co-workers (Experimental Neurology (1997) 146, 435-443) describe the neuronal delivery of Cu/Zn-SOD by fusion of the enzyme to the binding domain of tetanus toxin. None of these strategies, however, are particularly efficient at delivering the enzyme to the intracellular neuronal compartments where the enzyme can be effective.

In most eukaryotic species, two intracellular forms of superoxide dismutase exist: the Cu/Zn-SOD which is located within the cytoplasmic and nuclear compartments and manganese superoxide dismutase (Mn-SOD) which is located within the mitochondrial matrix. Human Mn-SOD is a tetrameric enzyme and is larger than the dimeric Cu/Zn-SOD. Several studies have shown that decreased Mn-SOD may be associated with one or more chronic diseases such as ovarian cancer (Nishida *et al.* (1995) Oncology Reports, 2, 643-646) and diabetes (L'Abbe *et al.*, (1994) Proc Soc Exp Biol Med, 207, 206-274). In addition, mice in which the Mn-SOD gene has been knocked out exhibit several novel pathogenic phenotypes including severe anaemia, degeneration of neurones in the basal ganglia and brainstem, and progressive motor disturbances characterised by weakness and rapid fatigue (Lebovitz *et al.*, (1996) Proc Natl Acad Sci USA, 93, 9782-9787). In addition these mice showed extensive damage to the neuronal mitochondria. Overexpression of Mn-SOD in cell lines and transgenic mice showed that damage and apoptosis of neurones under oxidative stress was markedly reduced (Keller *et al.*, (1998) Journal of Neuroscience, 18, 687-697). Mitochondrial damage was also reduced. These data showed that superoxide accumulation and subsequent mitochondrial damage play key roles in neuronal death induced by trauma both *in vitro* and *in vivo*. Delivery of agents which reduce the level of

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oxidative stress to neuronal cells may therefore reduce neuronal cell death and afford considerable therapeutic benefits.

5 Mn-SODs of bacterial origin such as that from *Bacillus stearothermophilus* or *B. caldotenax* consist of two subunits and are smaller than the human isoform which is a tetramer. *B. stearothermophilus* and *B. caldotenax* Mn-SOD also have considerably lower immunogenicity than the human isoform which is an advantage for continued therapeutic use. As enzymes for therapeutic applications, however, they suffer from similar drawbacks to
10 other SODs in that very little of the administered enzyme is retained within the tissues where it would be therapeutically beneficial.

15 The botulinum neurotoxins are a family of seven structurally similar, yet antigenically different, protein toxins whose primary site of action is the neuromuscular junction where they block the release of the transmitter acetylcholine. The action of these toxins on the peripheral nervous system of man and animals results in the syndrome botulism, which is characterised by widespread flaccid muscular paralysis (Shone (1986) in 'Natural Toxicants in Foods', Editor D. Watson, Ellis Harwood, UK). Each
20 of the botulinum neurotoxins consist of two disulphide-linked subunits; a 100 kDa heavy subunit which plays a role in the initial binding and internalisation of the neurotoxin into the nerve ending (Dolly et. al. (1984) Nature, 307, 457-460) and a 50 kDa light subunit which acts intracellularly to block the exocytosis process (McInnes and Dolly (1990) Febs Lett., 261, 323-326; de Paiva and Dolly (1990) Febs Lett., 277, 171-174). Thus it is
25 the heavy chains of the botulinum neurotoxins that impart their remarkable neuronal specificity.

30 Tetanus toxin is structurally very similar to botulinum neurotoxins but its primary site of action is the central nervous system where it blocks the release of inhibitory neurotransmitters from central synapses (Renshaw cells). As described for the botulinum toxins above, it is domains within

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the heavy chain of tetanus toxin that bind to receptors on neuronal cells.

5 The binding and internalisation (translocation) functions of the clostridial neurotoxin (tetanus and botulinum) heavy chains can be assigned to at least two domains within their structures. The initial binding step is energy-independent and appears to be mediated by one or more domains within the H_C fragment of the neurotoxin (C-terminal fragment of approximately 50kDa) (Shone *et al.* (1985), Eur. J. Biochem., 151, 75-82) while the translocation step is energy-dependent and appears to be
10 mediated by one or more domains within the H_N fragment of the neurotoxin (N-terminal fragment of approximately 50kDa).

15 Isolated heavy chains are non-toxic compared to the native neurotoxins and yet retain the high affinity binding for neuronal cells. Tetanus and the botulinum neurotoxins from most of the seven serotypes, together with their derived heavy chains, have been shown to bind a wide variety of neuronal cell types with high affinities in the nM range (e.g botulinum type B neurotoxin; Evans *et al.* (1986) Eur. J. Biochem. 154, 409-416).

20 Another key characteristic of the binding of these neurotoxins is that tetanus, botulinum A, B, C₁, D, E and F neurotoxins all appear to recognise distinct receptor populations, and collectively the clostridial neurotoxin heavy chains provide high affinity binding ligands that recognise a whole family of receptors that are specific to neuronal cells.

25 However, whilst it is known to provide a fusion of a SOD with a neurotoxin heavy chain, this fusion has been found to be ineffective for delivery of SOD to neuronal cells and inactive in *in vitro* assays for potential therapeutic activity.

30 It is an object of the invention to provide compositions and methods for delivery of SOD to neuronal cells. A further object is to provide

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compositions and methods for treatment or at least amelioration of conditions such as ischemic stroke.

Accordingly, the present invention provides a construct for delivering superoxide dismutase (SOD) to neuronal cells. In one aspect of the invention the construct consists of a SOD which has been combined with various functional protein domains to effect efficient targeting to the mitochondria within neuronal cells. The construct of specific embodiments of the invention, described in further detail below, contains the following elements:-

- a SOD which contains a leader sequence for targeting SOD to the mitochondria;
- a dimeric SOD which has low immunogenicity and high stability;
- a SOD which is linked to a domain that effects translocation across lipid membranes;
- a linkage between the SOD and the translocation domain that is cleaved within the neuronal cytosol; and
- a domain which selectively targets the construct to neuronal cells.

A first aspect of the invention thus provides a composition for delivery of superoxide dismutase (SOD) to neuronal cells, comprising:-

SOD; linked by a cleavable linker to
a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the SOD of the composition into the neuronal cell.

The linker is cleavable and thus, in use, after translocation of the SOD into the cell, the linker is cleaved to release SOD from the neuronal cell targeting domain. A suitable linker is a disulphide bridge between cysteine residues, one residue on the SOD and one residue on the neuronal cell targeting component, for example on the second domain. Another example of a linker is a site for a protease found in neuronal cells. In this way, the

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linker is such that it is cleaved within the cell, separating the SOD from the other translocated portions of the composition.

5 By SOD is meant a sequence that has superoxide dismutase activity, and may also comprise a further sequence or sequences conferring additional properties on that portion of the constructs. For example, the SOD optionally also includes a sequence targeting the SOD to mitochondria in a neuronal cell.

10 The SOD may be a hybrid of Mn-SOD and a sequence targeting the hybrid to mitochondria. The SOD may be of bacterial or human origin, or a derivative thereof, and may be comprised of sequences from more than one origin, provided that it has superoxide dismutase activity.

15 The first domain may suitably be selected from (a) neuronal cell binding domains of clostridial toxins; and (b) fragments, variants and derivatives of the domains in (a) that substantially retain the neuronal cell binding activity of the domains of (a). The second domain is suitably selected from (a) domains of clostridial neurotoxins that translocate polypeptide sequences
20 into cells, and (b) fragments, variants and derivatives of the domains of (a) that substantially retain the translocating activity of the domains of (a).

In an embodiment of the invention a construct comprises SOD linked by a disulphide bridge to a neuronal cell targetting component comprising a first
25 domain that binds to a neuronal cell and a second domain that translocates the SOD into the neuronal cell. This construct is made recombinantly as a single polypeptide having a cysteine residue on the SOD which forms a disulphide bridge with a cysteine residue on the second domain. The SOD is covalently linked, initially, to the second domain. Following expression
30 of this single polypeptide SOD is cleaved from the second domain leaving the SOD linked only by the disulphide bridge to the rest of the construct.

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A second aspect of the invention provides a pharmaceutical composition for treatment of oxidative damage to neuronal cells comprising a composition according to the invention with a pharmaceutically acceptable carrier. This composition may be used to deliver SOD to a neuronal cell, for example by administration of the composition by injection.

A third aspect of the invention provides a method of preparing a composition according to the invention, comprising chemically linking SOD, linker and neuronal cell targeting components. The SOD preferably is free of cysteine residues and the method preferably comprises treating the SOD with a cross-linker which will form a disulphide bridge with a cysteine residue on the neuronal cell targeting domain.

In a further embodiment of the third aspect of the invention, there is provided a method of making a composition according to the invention comprising expressing a DNA that codes for a polypeptide having SOD, a linker, a neuronal cell targeting component. The polypeptide may further comprise a purification sequence and the method may further comprise purifying the polypeptide using this sequence and then cleaving the polypeptide to remove the purification sequence to leave SOD, the linker and the neuronal cell targeting component.

By virtue of the combination of properties defined above, constructs of the invention are surprisingly efficient at transporting SOD to the mitochondria within neuronal cells. The ability of the superoxide dismutase to be translocated into the cytosol by virtue of the 'translocation domain' within the construct and the cleavage of the enzyme from the latter domain within the cell is key to this targeting efficiency. As such the construct of the invention has considerable therapeutic value in treating neuronal diseases which results from oxidative stress and has several advantages over previously described SOD formulations. Mitochondria within cells containing high levels of superoxide radicals are particularly sensitive to

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damage and the ability of the construct of the invention to target the SOD to these organelles offers considerable advantage in that the enzyme can act to remove the superoxide radicals where it is most required.

5 The construct of the invention may be used clinically in a variety of neuronal diseases which are caused or augmented by oxidative stress. Such conditions include ischaemic stroke, Parkinson's disease, Huntington's disease and motor neurone diseases. In the case of ischaemia/reperfusion injury caused by stroke or trauma, delivery of the construct of the invention to neurones of the hippocampus may afford considerable therapeutic
10 benefits by reducing neuronal damage and death. Other neuronal diseases where the underlying cause is oxidative stress would also benefit from the therapeutic effects of the construct of the invention.

15 In a preferred aspect of the invention, the SOD is a dimeric, manganese superoxide dismutase (Mn-SOD) which is of bacterial origin and has low immunogenicity and high stability.

The use of a bacterial Mn-SOD in constructs has a number of advantages compared to the use of the human Mn-SOD isoform:-
20

- the low immunogenicity of the bacterial Mn-SOD is advantageous where repeated administration of the construct is required, in which cases the induction of adverse host immune responses is reduced;
25 and

- the smaller size of constructs based on the dimeric bacterial Mn-SOD compared to human Mn-SOD (which is a tetramer) both reduces the likelihood of adverse immune responses and increases
30 the rate of diffusion of the construct to its target tissue.

In exercise of an example of the invention, a bacterial Mn-SOD of low

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immunogenicity is derived from either *B. stearothermophilus* (sequence as reported by Brock and Walker (1980) Biochemistry, 19, 2873-2882) or *B. caldotenax* (gene and amino acid sequence as defined by Chambers *et al.*, (1992) FEMS Microbiology Letters, 91, 277-284) to which a mitochondrial leader sequence has been fused to the N-terminus of the protein by recombinant technology. This Mn-SOD-leader hybrid is linked by a disulphide bridge to a translocation domain derived from a bacterial protein toxin, such as botulinum neurotoxin. The translocation domain, in turn is fused to a receptor binding domain derived from a clostridial neurotoxin (botulinum or tetanus). The construct is produced initially as a single polypeptide by recombinant technology and subsequently converted to the construct of the invention by selective cleavage with a proteolytic enzyme. To produce the construct of the invention, a loop motif containing a unique protease site (e.g amino acid sequences specifically cleaved by proteases such as factor Xa, enterokinase, thrombin) and a cysteine residue is introduced between the C-terminus of the Mn-SOD and the N-terminus of the translocation domain such that a disulphide bridge is formed between the Mn-SOD and the translocation domain. Subsequent cleavage of the protease site generates the active construct. The final construct, when analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis in the presence of a reducing agent (e.g. dithiothreitol), dissociates into two major bands, one corresponding to the superoxide dismutase enzyme and a second corresponding to a polypeptide which contains the neuronal binding and translocation domains. In the absence of a reducing agent this dissociation does not occur and the complex is observed as a single major band on the gels.

Modification of Mn-SOD from *B. stearothermophilus* by addition of a mitochondrial targeting sequence offers several advantages over the use of human Mn-SOD which contains its own mitochondrial leader sequence. Firstly, the *B. stearothermophilus* Mn-SOD has a high thermal stability and low immunogenicity which allows administration of several doses of the

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enzyme without provoking an immune response from the host that would reduces its efficacy. Secondly, the *B. stearothermophilus* Mn-SOD is a small dimeric enzyme unlike the human Mn-SOD which is a tetramer. Recombinant constructs containing the latter enzyme would therefore have to be considerably larger and more complex in their structure.

In an embodiment of the invention, a DNA encoding a construct of the invention is made up by fusion of following DNA fragments commencing at the 5' end of the gene:-

an oligonucleotide encoding a modified human mitochondrial leader sequence (amino acid sequence: MLSRAVCGTSRQLAPALGYLGSRQ (SEQ ID NO:10) or MLSRAVSGTSRQLAPALGYLGSRQ (SEQ ID NO:11);

an oligonucleotide encoding Mn-SOD from *B. stearothermophilus* (coding for the amino acid sequence as defined in Brock and Walker (1980) Biochemistry, 19, 2873-2882);

an oligonucleotide encoding a linker peptide which contains the thrombin protease cleavage site and a cysteine residue for disulphide bridge formation (peptide sequence: CGLVPAGSGP);

an oligonucleotide encoding a translocation domain derived from a botulinum neurotoxin (e.g. a DNA fragment coding for amino acid residues 449-871 of botulinum type A neurotoxin, or a DNA fragment coding for amino acid residues 441-858 of botulinum type B neurotoxin, or a DNA fragment coding for amino acid residues 440-864 of botulinum type F neurotoxin); and

an oligonucleotide encoding the receptor binding domain of a botulinum neurotoxin or tetanus neurotoxin (e.g. a DNA fragment

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coding for amino acid residues 872-1296 of botulinum type A neurotoxin, or a DNA fragment coding for amino acid residues 859-1291 of botulinum type B neurotoxin, or a DNA fragment coding for amino acid residues 865-1278 of botulinum type F neurotoxin, or a DNA fragment coding for amino acid residues 880-1315 of tetanus neurotoxin).

The above DNA fragments may be obtained and constructed by standard recombinant DNA methods. Expression and purification of the assembled construct may be obtained with a variety of suitable expression hosts, e.g. *Escherichia coli*, *Bacillus subtilis*.

The translocation domain and neuronal binding domain of the construct may also be derived from combination of different clostridial neurotoxins. For example, the construct of the invention may contain a translocation domain derived from botulinum type F neurotoxin and a binding domain derived from botulinum type A neurotoxin.

A construct of the invention may be produced using protein chemistry techniques. Mn-SOD derived from *B. stearothermophilus* to which a mitochondrial leader sequence has been fused to the N-terminus of the protein by recombinant technology is modified with a heterobifunctional cross-linking reagent such as N-succinimidyl 3-[2-pyridyldithio] propionate (SPDP). The chemically modified enzyme is then combined to a cell targetting domain which contains the binding and translocation functional domains. The latter may be produced by recombinant technology or purified from the neurotoxins of *Clostridium botulinum* or *Clostridium tetani* by established methods. Chemical coupling of the SPDP-treated Mn-SOD may be accomplished using a free cysteine residue on the polypeptide containing the binding and translocation domains to give a construct of the invention.

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Constructs of the invention may be introduced into either neuronal or non-neuronal tissue using methods known in the art. By subsequent specific binding to neuronal cell tissue, the targeted construct will exert its therapeutic effects. Alternatively, the construct may be injected near a site requiring therapeutic intervention, e.g. intrathecal or intracranial injection close to a site of trauma or disease.

The construct of the invention may also be administered with other agents which enhance its delivery to its target tissue. An example of such an agent is one which assists the passage of the construct of the invention through the blood-brain barrier to the central nervous system. The construct of the invention may also be administered in formulations with other therapeutic agents or drugs.

The dosage required for the construct of the invention will depend upon the application and could vary between $1\mu\text{g/kg}$ to 100mg/kg of body weight. The construct of the invention may be produced as a suspension, emulsion, solution or as a freeze dried powder depending on the application and properties of the release vehicle and its therapeutic contents. The construct of the invention may be resuspended or diluted in a variety of pharmaceutically acceptable liquids depending on the application.

"Clostridial neurotoxin" means a neurotoxin corresponding to tetanus neurotoxin or one of the seven botulinum neurotoxin serotypes (type A, B, C₁, D, E, F or G).

"Bind" in relation to the clostridial binding fragments, means the interaction between the clostridial fragment and one or more cell surface receptors or markers which results in localisation of the binding fragment or construct in the vicinity of the cell.

"Binding domain" of botulinum or tetanus neurotoxins means a domain of

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the toxin which retains the property of being able to bind the receptors on neuronal cells in a similar manner to the intact neurotoxin and encompasses native domains and fragments, variants and derivatives that retain this binding function. This property of the binding domain can be assessed in competitive binding assays. In such assays, radiolabelled neurotoxin (e.g. botulinum type A neurotoxin) is contacted with neuronal cells in the presence of various concentrations of non-radiolabelled fragment representing the 'binding domain' of the neurotoxin. The ligand mixture is incubated with the cells, at low temperature (0-3°C) to prevent ligand internalisation, during which competition between the radiolabelled neurotoxin and non-labelled 'binding domain' fragment may occur. In such assays when the unlabelled ligand used is binding domain of botulinum type A neurotoxin (residues 872-1296), the radiolabelled botulinum type A neurotoxin will be displaced from the neuronal cell receptors as the concentration of its non-labelled 'binding domain' is increased. The competition curve obtained in this case will therefore be representative of the behaviour of a 'binding domain' fragment being able to bind the receptors on neuronal cells in a similar manner to the intact neurotoxin. This property of the binding domain may be used to identify other suitable protein domains which have the desired binding properties. Examples of binding domains derived from clostridial neurotoxins are as follows:-

Botulinum type A neurotoxin	- amino acid residues (872 - 1296)
Botulinum type B neurotoxin	- amino acid residues (859 - 1291)
Botulinum type C neurotoxin	- amino acid residues (867 - 1291)
Botulinum type D neurotoxin	- amino acid residues (863 - 1276)
Botulinum type E neurotoxin	- amino acid residues (846 - 1252)
Botulinum type F neurotoxin	- amino acid residues (865 - 1278)
Botulinum type G neurotoxin	- amino acid residues (864 - 1297)
Tetanus neurotoxin	- amino acid residues (880 - 1315)

"Translocation domain" means a domain or fragment of a protein which effects transport of itself and/or other proteins and substances across a

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membrane or lipid bilayer and encompasses native domains and fragments, variants and derivatives that retain this binding function. The latter membrane may be that of an endosome where translocation will occur during the process of receptor-mediated endocytosis. Translocation domains can frequently be identified by the property of being able to form measurable pores in lipid membranes at low pH (Shone *et al.* (1987) Eur J. Biochem. 167, 175-180). The latter property of translocation domains may thus be used to identify other protein domains which could function as the translocation domain within the construct of the invention. Examples of translocation domains derived from bacterial neurotoxins are as follows:-

Botulinum type A neurotoxin	– amino acid residues (449 - 871)
Botulinum type B neurotoxin	– amino acid residues (441 - 858)
Botulinum type C neurotoxin	– amino acid residues (442 - 866)
Botulinum type D neurotoxin	– amino acid residues (446 - 862)
Botulinum type E neurotoxin	– amino acid residues (423 - 845)
Botulinum type F neurotoxin	– amino acid residues (440 - 864)
Botulinum type G neurotoxin	– amino acid residues (442 - 863)
Tetanus neurotoxin	– amino acid residues (458 - 879)

“Translocation” in relation to translocation domain, means the internalisation events which occur after modified clostridial binding fragments bind to the cell surface. These events lead to the transport of substances into the cytosol of neuronal cells.

“Unique protease site” means a protease site incorporated into the construct such that the molecule may be proteolysed at pre-determined sites by a selected protease. The specificity of these proteases is such that cleavage to other parts of the construct does not occur. Examples of unique protease sites are the amino acid sequences cleaved by proteases such as: thrombin, factor Xa, enterokinase.

A fourth aspect of the invention provides a composition for delivery of a

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therapeutic agent to neuronal cells, comprising:-

the therapeutic agent; linked by a cleavable linker to
a neuronal cell targeting component, comprising a first domain that
binds to a neuronal cell and a second domain that translocates the
therapeutic agent of the composition into the neuronal cell.

Thus, in use, after translocation of the therapeutic agent into the cell, the
linker is cleaved to release the therapeutic agent from the neuronal cell
targeting domain. Other optional and preferred embodiments of the fourth
aspect of the invention are as for the first-third aspects of the invention.

A fifth aspect of the invention provides a polypeptide comprising a
bacterial SOD, or derivative thereof, and a sequence for targeting the
polypeptide to a mitochondria, such as a human mitochondria. The
polypeptide may be chemically obtained by synthesis of otherwise or may
be a fusion protein, obtained for example by expression of a nucleotide
coding for the polypeptide.

The invention hence also provides, in a sixth aspect, a nucleotide encoding
the polypeptide of the fifth aspect and in a seventh aspect a vector
comprising the nucleotide of the sixth aspect. Also provided in an eighth
aspect is a method of making a polypeptide according to the fifth aspect
comprising expressing the nucleotide sequence of the sixth aspect. In a
ninth aspect is provided a cell comprising the nucleotide sequence of the
sixth aspect or the vector of the seventh.

There now follows description of specific embodiments of the invention
illustrated by drawings in which:-

Fig. 1 shows schematic examples of novel Mn-SODs derived from
B. stearothermophilus and *B. caldotenax*. Two examples of mitochondrial
leader sequences are shown. In one example, a cysteine residue at position

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7 has been mutated to a serine residue. This change enables the production of the construct of the invention without the formation of disulphide bridges in undesirable positions;

5 Figure 2 shows schematic examples of novel Mn-SOD fusion proteins showing the use of peptides and proteins to facilitate purification of the enzyme from the production strain. Various protein and peptide tags (such as histidine-6, S-peptide, maltose-binding protein, calmodulin-binding protein) may be fused to the Mn-SOD to allow rapid purification by affinity
10 chromatography methods. Unique protease sites are incorporated between the purification tag and the Mn-SOD to enable removal of the tag after purification. Protein and peptide tags may be removed by treatment of the fusion protein with the relevant specific protease (e.g. factor Xa, thrombin, enterokinase);

15 Fig. 3 shows a recombinant Mn-SOD construct of the invention. From the N-terminus of the protein, the construct consists of the following components:- (1) a mitochondrial leader (targeting) sequence, (2) a Mn-superoxide dismutase, (3) a loop which contains a unique protease site and
20 which allows disulphide bridge formation, (4) a translocation domain, (5) a neuronal targeting domain. The construct is produced as a single polypeptide; subsequent cleavage with a protease specific for the 'unique protease site' contained within the loop region generates the di-chain construct. Purification tags could added to the constructs as exemplified
25 in Figure 3;

30 Fig. 4 shows the production of a Mn-SOD construct by chemical methods. The method uses a recombinant Mn-SOD, purified as described in Example 1 and coupled to a polypeptide containing the translocation and binding domains as described in Example 4; and

Fig. 5 shows the results of an example to demonstrate the protective

- 17 -

effects of a construct of the invention on NG108 cells subjected to oxidative stress by the addition of 50 μ M duroquinone for four hours.

The application is also accompanied by a sequence listing in which:-

5

SEQ ID NO: 1 shows the amino acid sequence of Mn-SOD from *B. caldotenax*;

10

SEQ ID NO: 2 shows the amino acid sequence of Mn-SOD from *B. stearothermophilus*;

15

SEQ ID NO: 3 shows the amino acid sequence of a construct of the invention comprising Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype A;

20

SEQ ID NO: 4 shows the amino acid sequence of a construct of the invention comprising Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype B;

25

SEQ ID NO: 5 shows the amino acid sequence of a construct of the invention comprising Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype F;

30

SEQ ID NO: 6 shows the amino acid sequence of a construct of the invention comprising a mitochondrial leader sequence from human Mn-SOD, Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype A;

- 18 -

SEQ ID NO: 7 shows the amino acid sequence of a construct of the invention comprising a mitochondrial leader sequence from human Mn-SOD, Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype B;

SEQ ID NO: 8 shows the amino acid sequence of a construct of the invention comprising a mitochondrial leader sequence from human Mn-SOD, Mn-SOD from *B. stearothermophilus*, a linker that can be cleaved by thrombin, and a heavy chain derived from botulinum neurotoxin serotype F; and

SEQ ID NO: 9 shows the amino acid sequence for a polypeptide comprising a mitochondrial leader sequence from human Mn-SOD and Mn-SOD from *B. stearothermophilus*;

SEQ ID NO: 10 shows the amino acid sequence of a modified human mitochondrial leader sequence; and

SEQ ID NO: 11 shows an amino acid sequence of a modified human mitochondrial leader sequence.

Example 1.

Production and purification of novel *B. stearothermophilus* Mn-SOD containing a mitochondrial leader sequence.

Standard molecular biology protocols were used for all genetic manipulations (eg. Sambrook *et al.* 1989, Molecular Cloning a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). A synthetic gene encoding the mitochondrial targeting sequence (amino acids 1-27) of the human Mn-SOD gene was cloned as

- 19 -

an *NdeI* - *Bam* HI fragment into an expression vector so that the transcriptional start corresponds to the ATG codon within the *NdeI* site. The Mn-SOD gene from *B. stearrowthermophilus* or *B. caldotenax* was amplified using PCR to give a *Bgl*III site corresponding to the leucine amino acid at position 5 at the 5' end, and a *Bam*HI site outside the stop codon at the 3' end. This *Bs*III-*Bam*HI fragment was cloned into the expression vector carrying the mitochondrial targeting sequence (digested *Bam*HI) to generate "in-frame" gene fusions. In addition to the wild type mitochondrial targeting sequence, a variant was constructed in which the cysteine at position 7 was changed to serine

The recombinant Mn-SOD expressed in pET28a were produced with amino-terminal histidine (6 His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Ni^{2+} charged column or an anti-T7 immunoaffinity column (Smith *et al.* 1988, *Journal of Biological Chemistry*, 263: 7211-7215). Incorporation of a factor Xa protease cleavage between the peptide tag and the expressed Mn-SOD allowed this to be removed after purification. Briefly, cultures of *E.coli* BL21 (DE3) pET28a-Mn-SOD were grown in Terrific broth-kanamycin ($30 \mu\text{gml}^{-1}$) to an $\text{OD}_{600 \text{ nm}}$ of 2.0, and protein expression was induced by the addition of $500 \mu\text{M}$ IPTG for approximately 2 h. Cells were lysed by freeze/thaw followed by sonication, lysates cleared by centrifugation and supernatants loaded onto an anion exchange column (MonoQ™ column on a Fast Protein Liquid Chromatography system; Pharmacia Biotech, Uppsala, Sweden). Eluted recombinant Mn-SOD was then desalted and further purified by affinity chromatography on a chelating sepharose column charged with Ni^{2+} (Pharmacia Biotech, Uppsala, Sweden). After loading proteins onto the column and subsequent washing, the purified Mn-SOD was eluted with imidazole. All buffers used were as specified by the manufacturer.

A 'maltose binding protein' purification tag was also employed for the purification of some batches of Mn-SOD. The use of this system is

- 20 -

described in detail in New England Biolabs Instruction Manual "Protein Fusion and Purification System" (ver 3.02).

5 Other tags and protease cleavage site may also be incorporated into the sequence to facilitate purification of Mn-SOD as exemplified in Figure 2.

10 For purification of a novel *B. stearothermophilus* Mn-SOD which was not conjugated to protein purification tag, the following procedure was used. After harvesting, cells were broken by high pressure homogenisation crude extracts were clarified by centrifugation and batch purified on DE-23 cellulose. The fraction eluted with 0.4M NaCl contained the Mn-SOD. This fraction was then further purified by various chromatographic media using the following sequence:-

15 DEAE-Sepharose ion exchange chromatography at pH 8.0; elution of the Mn-SOD with a NaCl gradient;

hydroxylapatite chromatography at pH 6.8; elution of Mn-SOD with a phosphate gradient at pH 6.8;

20 ion exchange chromatography on Q-Sepharose at pH 7.5; elution with a NaCl gradient; and

gel filtration on Sephacryl S-200.

25 The purified Mn-SOD may be dialysed against Hepes buffer (0.1M, pH7.4) containing 0.15M NaCl and stored at -80°C.

Example 2.

30 Preparation and purification of a recombinant Mn-SOD construct of the invention.

- 21 -

Standard molecular biology protocols were used for all genetic manipulations (*eg.* Sambrook *et al.* 1989, Molecular Cloning a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Various DNA fragments of the construct were generated using Recursive PCR reactions (Prodromou & Pearl 1992, *Protein Engineering*, 5: 827-829) using self-priming oligonucleotides containing the desired sequence. For the expression of clostridial neurotoxin fragments the codon bias and GC/AT base ratio was adjusted for ease of expression in *E. coli*. Fragments were cloned sequentially into pLitmus 38 (New England Biolabs, Inc., Beverly, MA) to assemble the entire gene. Constructs for expression were sub-cloned into pET28b (Novagen Inc., Madison, WI) replacing the *EcoR1-HindIII* fragment. The ligation reactions were transformed into *E. coli* DH5 α (Life Technologies Inc., Gaithersburg, MD). Plasmid DNA was amplified, purified and screened for the presence of the appropriate sequence (Ausubel *et al.* 1989, Current Protocols in Molecular Biology, John Wiley & Sons, New York). Gene constructions confirmed as possessing the correct sequences were then transformed into the expression host *E. coli* BL21 (DE3) (Studier & Moffatt 1986, *Journal of Molecular Biology*, 189: 113-130).

The recombinant constructs expressed in pET28 were produced with amino-terminal histidine (6 His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Ni²⁺ charged column or an anti-T7 immunoaffinity column (Smith *et al.* 1988, *Journal of Biological Chemistry*, 263: 7211-7215). Incorporation of a factor X protease cleavage between the peptide tags and the expressed Mn-SOD Constructs allowed these to be removed after purification. Briefly, cultures of *E. coli* BL21 (DE3) pET28-Mn-SOD Construct were grown in Terrific broth-kanamycin (30 μ gml⁻¹) to an OD₆₀₀ nm of 2.0, and protein expression was induced by the addition of 500 μ M IPTG for approximately 2 h. Cells were lysed by freeze/thaw followed by sonication, lysates cleared by centrifugation and supernatants loaded onto an anion exchange column (MonoQ™ column on

- 22 -

a Fast Protein Liquid Chromatography system; Pharmacia Biotech, Uppsala, Sweden). Eluted recombinant Mn-SOD Construct was then desalted and further purified by affinity chromatography on a chelating sepharose column charged with Ni^{2+} (Pharmacia Biotech, Uppsala, Sweden). After loading proteins onto the column and subsequent washing, the purified Construct was eluted with imidazole. All buffers used were as specified by the manufacturer.

A 'maltose binding protein' purification tag was also employed for the purification some batches of Mn-SOD Constructs. The use of this system is described in detail in New England Biolabs Instruction Manual "Protein Fusion and Purification System" (ver 3.02).

It would also be evident to anyone skilled in the art that other tags and protease cleavage site may also be incorporated into the sequence to facilitate purification as exemplified in Figure 3.

The amino sequences of several recombinant Mn-SOD constructs are shown in the sequence listing.

Example 3.

Preparation of botulinum heavy chains by chemical methods.

The various serotypes of the clostridial neurotoxins may be prepared and purified from various toxigenic strains of *Clostridium botulinum* and *Clostridium tetani* by methods employing standard protein purification techniques as described previously (Shone and Tranter 1995, Current Topics in Microbiology, 194, 143-160; Springer). Samples of botulinum neurotoxin (1mg/ml) are dialysed against a buffer containing 50mM Tris-HCl pH 8.0, 1M NaCl and 2.5M urea for at least 4 hours at 4°C and then made 100mM with dithiothreitol and incubated for 16h at 22°C. The cloudy

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solution which contains precipitated light chain is then centrifuged at 15000 x g for 2 minutes and the supernatant fluid containing the heavy chain retained and dialysed against 50mM Hepes pH 7.5 containing 0.2M NaCl and 5mM dithiothreitol for at least 4 hours at 4°C. The dialysed heavy chain is centrifuged at 15000 x g for 2 minutes and the supernatant retained and dialysed thoroughly against 50mM Hepes pH 7.5 buffer containing 0.2M NaCl and stored at -70°C. The latter procedure yields heavy chain >95% pure with a free cysteine residue which can be used for chemical coupling purposes. Biological (binding) activity of the heavy chain may be assayed as described in Example 5.

The heavy chains of the botulinum neurotoxins may also be produced by chromatography on QAE Sephadex as described by the methods in Shone and Tranter (1995) (Current Topics in Microbiology, 194, 143-160; Springer).

Example 4

Production of Mn-SOD constructs by chemical methods

B. stearothermophilus Mn-SOD fused to a mitochondrial leader sequence was purified as described in Example 1. The Mn-SOD was chemically modified by treatment with a 3-5 molar excess of N-succinimidyl 3-[2-pyridyldithio] propionate (SPDP) in 0.05M Hepes buffer pH 7.0 containing 0.1M NaCl for 60 min at 22°C. The excess SPDP was removed by dialysis against the same buffer at 4°C for 16h. The substituted SOD was then mixed in a 1:2.5 molar ratio with heavy chain purified from *Clostridium botulinum* type A neurotoxin purified as described in Example 3 and incubated at 4°C for 16h. During the incubation period the Mn-SOD was conjugated to the botulinum heavy chain fragment by free sulphhydryl groups (see Figure 4). After incubation, the Mn-SOD-construct was purified by gel filtration chromatography on Sephadex G200.

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Constructs of the invention may also be formed by the above method using polypeptides containing the translocation and binding domains that have been produced by recombinant technology as outlined in Example 2.

5 **Example 5.**

**Assay of the biological activity of constructs -
demonstration of high affinity binding to neuronal cells.**

10 Clostridial neurotoxins may be labelled with ¹²⁵-iodine using chloramine-T and its binding to various cells assessed by standard methods such as described in Evans *et al.* 1986, Eur J. Biochem., 154, 409 or Wadsworth
15 *et al.* 1990, Biochem. J. 268, 123). In these experiments the ability of Mn-SOD constructs to compete with native clostridial neurotoxins for receptors present on neuronal cells or brain synaptosomes was assessed. All binding experiments were carried out in binding buffers. For the botulinum neurotoxins this buffer consisted of: 50mM HEPES pH 7.0, 30mM NaCl, 0.25% sucrose, 0.25% bovine serum albumin. For tetanus
20 toxin, the binding buffer was: 0.05M tris-acetate pH 6.0 containing 0.6% bovine serum albumin. In a typical binding experiment the radiolabelled clostridial neurotoxin was held at a fixed concentration of between 1-20nM. Reaction mixtures were prepared by mixing the radiolabelled toxin with various concentrations of unlabelled neurotoxin or construct. The reaction mixture were then added to neuronal cells or rat brain
25 synaptosomes and then incubated at 0-3°C for 2hr. After this period the neuronal cells of synaptosomes were washed twice with binding ice-cold binding buffer and the amount of labelled clostridial neurotoxin bound to cells or synaptosomes was assessed by γ -counting. In an experiment using an Mn-SOD construct which contained the binding domain from botulinum
30 type A neurotoxin, the construct was found to compete with ¹²⁵I-labelled botulinum type A neurotoxin for neuronal cell receptors in a similar manner to unlabelled native botulinum type A neurotoxin. These data showed that

- 25 -

the construct had retained binding properties of the native neurotoxin.

Example 6

5 **Assay of the biological activity of constructs – measurement of the Mn-SOD activity.**

Mn-SOD activity in samples and constructs was measured by a
modification (Brehm *et al.* (1991) Appl. Microbiol. Biotechnol., 36,358-363)
10 of the procedure described by McCord and Fridovich (J. Biol. Chem.
(1969), 244, 6049-6055). Aliquots (20 μ l) of samples or constructs
containing Mn-SOD were added to 1ml of 0.05M potassium phosphate
buffer pH 7.5 containing 1×10^{-4} M EDTA, 2.5×10^{-5} M ferricytochrome C
and 7×10^{-3} M sodium xanthine in a thermostatted cuvette at 30°C.
15 Sufficient xanthine oxidase was added to produce a rate of reduction of the
ferricytochrome C at 550nm of approx. 0.1 absorbance units/minute in the
absence of Mn-SOD. Under these conditions the amount Mn-SOD that was
required to reduce the rate of reduction of ferricytochrome C by 50% was
defined as one unit of activity.

20

Using such assays the Mn-SOD activity within constructs was assessed.

Example 7

25 **Demonstration of the targeting of Mn-SOD to the mitochondria of neuronal cells by constructs of the invention**

Mn-SOD construct containing the translocation and targeting domains
derived from botulinum type A neurotoxin was incubated at various
30 concentrations (0.01-10 μ M final concentration) with a neuroblastoma cell
line NG108. Incubations were carried out over a 6h period or overnight at
37°C. In some experiments, construct radiolabelled with 125 I iodine was

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us d. After incubation with the construct cells, were removed from culture
flasks by gentle scraping and centrifuged at 200 x g. Cells were then
resuspended in breaking buffer (0.6M mannitol, 20mM hepes pH 7.4 and
1mM phenylmethylsulphonyl chloride) and homogenised in a Dounce
homogeniser. The homogenate was centrifuged at 200 x g for 5min and
then the supernatant fluid recovered and centrifuged at 8000 x g for
10min. The 200 x g pellet (nuclear fraction) were pooled and resuspended
in phosphate buffered saline. The 8000 x g pellets (mitochondrial fraction)
were also pooled and resuspended in phosphate buffered saline. The
supernatant fluid was saved and used to represent the cytosolic fraction.

Analysis of the sub-cellular distribution of Mn-SOD was carried out by
Western blot analysis and, where radiolabelled construct was used, by
analysis of the ¹²⁵I-labelled construct components by γ -counting and by
autoradiography of cell fractions which had been separated by
electrophoresis on SDS-polyacrylamide gels. For Western blot analysis,
proteins in the cell fractions were separated by electrophoreses on SDS-
polyacrylamide gels and then transferred to nitrocellulose membrane as
described previously (Towbin *et al.* Proc.(1979) Natl. Acad. Sci. USA, 76,
4350). The presence of Mn-SOD in protein bands on nitrocellulose
membranes was assessed by incubation with rabbit anti- Mn-SOD antibody
followed by washing and incubation with anti-rabbit peroxidase conjugate.
Addition of peroxidase substrates (3,3',5,5'- tetramethyl benzidine and
H₂O₂) allowed visualisation and quantitation of the Mn-SOD in the various
sub-cellular protein fractions. An enhanced chemiluminescence system
(Amersham International) was also used in some experiments to increase
the sensitivity.

Example 8.

Formulation of the Mn-SOD construct for clinical use.

(
- 27 -

In a formulation of the Mn-SOD construct for clinical use, recombinant Mn-SOD construct would be prepared under current Good Manufacturing Procedures. The construct would be transferred, by dialysis, to a solution to give the product stability during freeze-drying. Such a formulation may contain Mn-SOD construct (10 mg/ml) in 5mM HEPES buffer (pH 7.2), 50mM NaCl, 1% lactose. The solution, after sterile filtration, would be aliquotted, freeze-dried and stored under nitrogen at -20°C.

Example 9.

Use of an Mn-SOD construct to treat stroke.

In a typical case of a middle aged or elderly man diagnosed as suffering from stroke, treatment with an Mn-SOD construct would begin immediately, ideally within 6 hours of the stroke occurring. Doses of the Mn-SOD construct (e.g. 100mg) reconstituted in a sterile saline solution would be administered intravenously. Further doses of the construct would be administered daily for 5-10 days. Such a patient would be expected to display reduced levels of ischaemia/reperfusion damage as assessed by magnetic resonance imaging compared to a similarly affected patient receiving no treatment. Relative improvements to muscle strength and co-ordination (MRC motor score) would be expected to be observed over the subsequent 12 month period.

Example 10.

Method to Demonstrate Neuroprotection of Cells by MnSOD-Heavy Chain Conjugates

Neuroblastoma cell line NG108-15 (*Nature* (1998) 336:p185) were seeded at a density of 3×10^4 cells/ml in 96 well microtitre plates coated with poly-D-lysine. Plates were grown for 3 days at 37°C in a CO₂ incubator (5% CO₂).

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95% air). MnSOD or leader-MnSOD conjugates were prepared with purified heavy chain of botulinum neurotoxin serotype A (BoNT/A HC) as described. The concentration of SOD was estimated and the conjugate diluted to give the specified amount of conjugate in a total volume of 200 μ l serum free medium. Conjugate was added to wells in the presence or absence of 56mM KCl, 2mM CaCl₂. The cells were incubated with conjugate for 1 hour. The conjugate was replaced with either serum free medium or serum free medium containing 50 μ M duroquinone and incubated at 37°C for 4 hours in the CO₂ incubator to induce oxidative stress. The media was removed after 4 hours and replaced with the dye 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.25mg/ml in serum free medium and incubated for 2 hours (according to the method of Mattson, MP, *et al* (1995) *Methods in Cell Biology* 46:187-216) The conversion of MTT to formazan dye crystals has been shown to be related to mitochondrial respiratory chain activity (Musser, DA, and Oseroff, AR (1994) *Journal of Immunology* 59:621-626). MTT was removed and crystals solubilised with dimethylsulfoxide (DMSO). Absorbance at 570nm was measured using a Labsystems Multiskan Plus MkII spectrophotometer and the results shown in Figure 5.

Figure 5 shows that a construct of the invention was able to protect neuronal cells against the mitochondrial - focused oxidative stress produced by duroquinone.

Example 11.

Preparation and purification of recombinant MnSOD and leader sequence MnSOD

Standard molecular biology protocols were used for all genetic manipulations (Sambrook *et al* 1989, Molecular cloning; A laboratory manual. Second Edition, Cold Spring Harbor Laboratory Press, New York.).

- 29 -

The MnSOD gene from either *B. stearothermophilus* or *B. caldopenax* was amplified by PCR to engineer a *Bam*HI site (resulting in the replacement of nucleotides 1-15). A synthetic oligonucleotide corresponding to the mitochondrial leader sequence of human MnSOD (bases 1-81 of the human gene) was subcloned into the *Bam*HI site to generate leader-MnSOD. PCR was used to add a Factor Xa cleavage site immediately adjacent to the methionine at the start of the leader sequence. Similarly a Factor Xa cleavage site was engineered immediately adjacent to the methionine at the start of the native MnSOD gene. Constructs were sequenced to confirm the presence of the correct sequence. Constructs for expression were subcloned into the expression vector pET28a (Novagen Inc, Madison, WI) as an *Eco*RI fragment and the orientation of the fragments checked. Clones with confirmed sequences were used to transform expression host *E. coli* BL21 (DE3) (Studier and Moffatt 1986 *Journal of Molecular Biology* 189:113-130). Examples 2 and 3 above provide detailed methods.

The recombinant proteins expressed from pET28a contain amino-terminal histidine (6-His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Cu^{2+} charged metal chelate column or an anti-T7 immunoaffinity column. Incorporation of the Factor Xa site between the peptide tags and the start of either MnSOD or leader MnSOD allows the precise removal of the peptide tags after purification. Cultures of *E. coli* BL21(DE3) pET28a-MnSOD or BL21(DE3) pET28a-leader-MnSOD were grown in Terrific Broth containing 30 $\mu\text{g/ml}$ kanamycin and 0.5% (w/v) glucose to an OD_{600} of 2.0 and protein expression was induced with 500 μM IPTG for 2 hours. Cells were lysed by sonication, cell debris pelleted by centrifugation and the supernatant loaded onto a metal chelate column charged with Cu^{2+} (Amersham-Pharmacia Biotech, Uppsala, Sweden). After loading proteins on the column and washing, proteins were eluted using imidazole. All buffers were used as specified by manufacturers. Factor Xa cleavage of the eluted protein was carried out according to manufacturers instructions.

- 30 -

The invention thus provides constructs and methods for delivery of SOD to neuronal cells.

CLAIMS

1. A composition for delivery of superoxide dismutase (SOD) to neuronal cells, comprising:-

5 SOD; linked by a cleavable linker to
a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the SOD of the composition into the neuronal cell.

10 2. A composition according to Claim 1 for delivery of SOD to mitochondria of neuronal cells wherein the SOD comprises a sequence targeting the SOD to mitochondria in the neuronal cell.

15 3. A composition according to Claim 2 wherein the SOD is a hybrid of Mn-SOD and a sequence targeting the hybrid to mitochondria.

4. A composition according to Claim 2 or 3 wherein the mitochondria targeting sequence is derived from human Mn-SOD.

20 5. A composition according to any of Claims 1-4 wherein the SOD is bacterial SOD or is derived therefrom.

25 6. A composition according to any of Claims 1 to 5 wherein the first domain is selected from (a) neuronal cell binding domains of clostridial toxins; and (b) fragments, variants and derivatives of the domains in (a) that substantially retain the neuronal cell binding activity of the domains of (a).

30 7. A composition according to any Claims 1 to 6 wherein the second domain is selected from (a) domains of clostridial neurotoxins that translocate polypeptide sequences into cells, and (b) fragments, variants and derivatives of the domains of (a) that substantially retain the

- 32 -

translocating activity of the domains of (a).

8. A composition according to any of Claims 1 to 7 wherein the linker is a disulphide bridge.

9. A pharmaceutical composition for treatment of oxidative damage to neuronal cells comprising a composition according to any of Claims 1 to 8 and a pharmaceutically acceptable carrier.

10. A method of delivering SOD to a neuronal cell comprising administering a composition according to Claim 9.

11. A method according to Claim 10 comprising injecting the composition.

12. A method of making a composition according to any of Claims 1 to 8 comprising chemically linking SOD, a linker and a neuronal cell targeting component.

13. A method of making a composition according to any of Claims 1 to 8 comprising expressing a DNA that codes for a polypeptide having SOD activity, a linker, and a neuronal cell targeting component.

14. A method according to claim 13 wherein the polypeptide further comprises a purification sequence and the method further comprises purifying the polypeptide and then cleaving the polypeptide to remove the purification sequence to leave SOD, the linker and the neuronal cell targeting component.

15. A composition for delivery of a therapeutic agent to neuronal cells, comprising:-

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the therapeutic agent; linked by a cleavable linker to a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the therapeutic agent of the composition into the neuronal cell.

5

16. A polypeptide comprising a bacterial SOD or derivative thereof and a sequence for targeting the polypeptide to a human mitochondria.

10

17. A polypeptide according to Claim 16 wherein the SOD is from *Bacillus*.

18. A polypeptide according to Claim 16 or 17 which is a fusion protein.

15

19. A nucleotide encoding the polypeptide of any of Claims 16-18.

20. A vector comprising the nucleotide of Claim 19.

20

21. A method of making a polypeptide according to any of Claims 16-18 comprising expressing the nucleotide sequence of Claim 19.

22. A cell comprising the nucleotide sequence of Claim 19 or the vector of Claim 20.

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FIG. 1

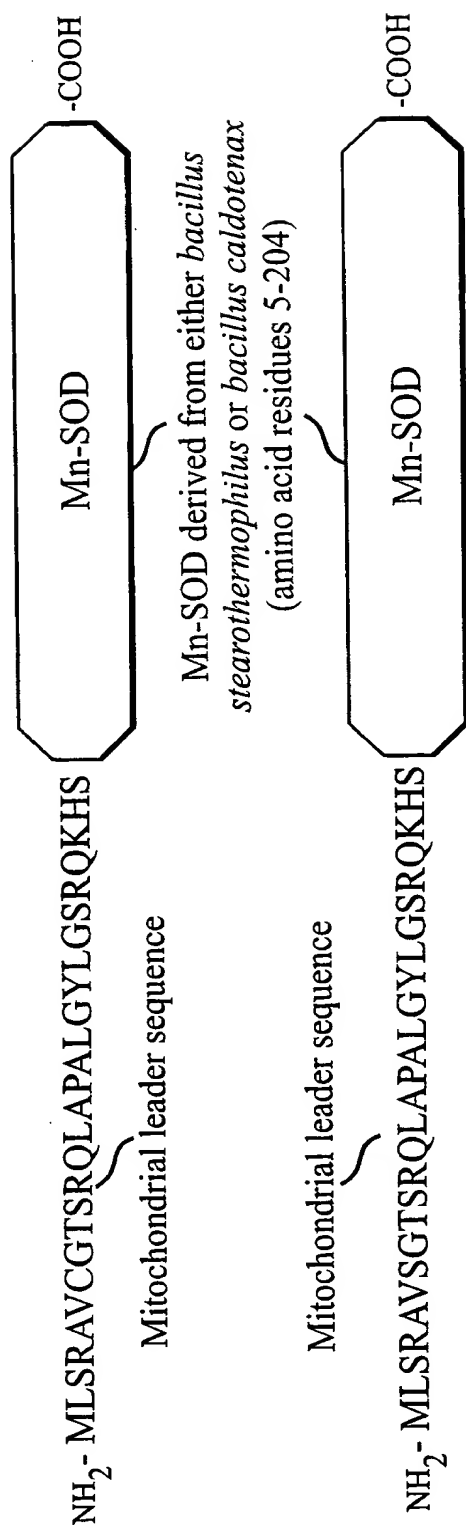
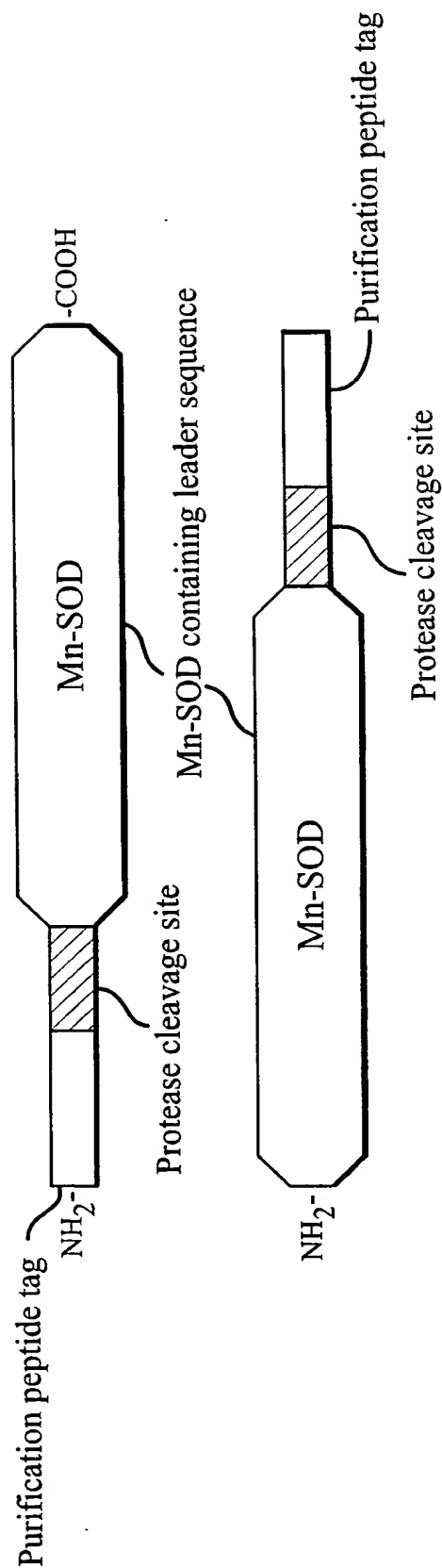
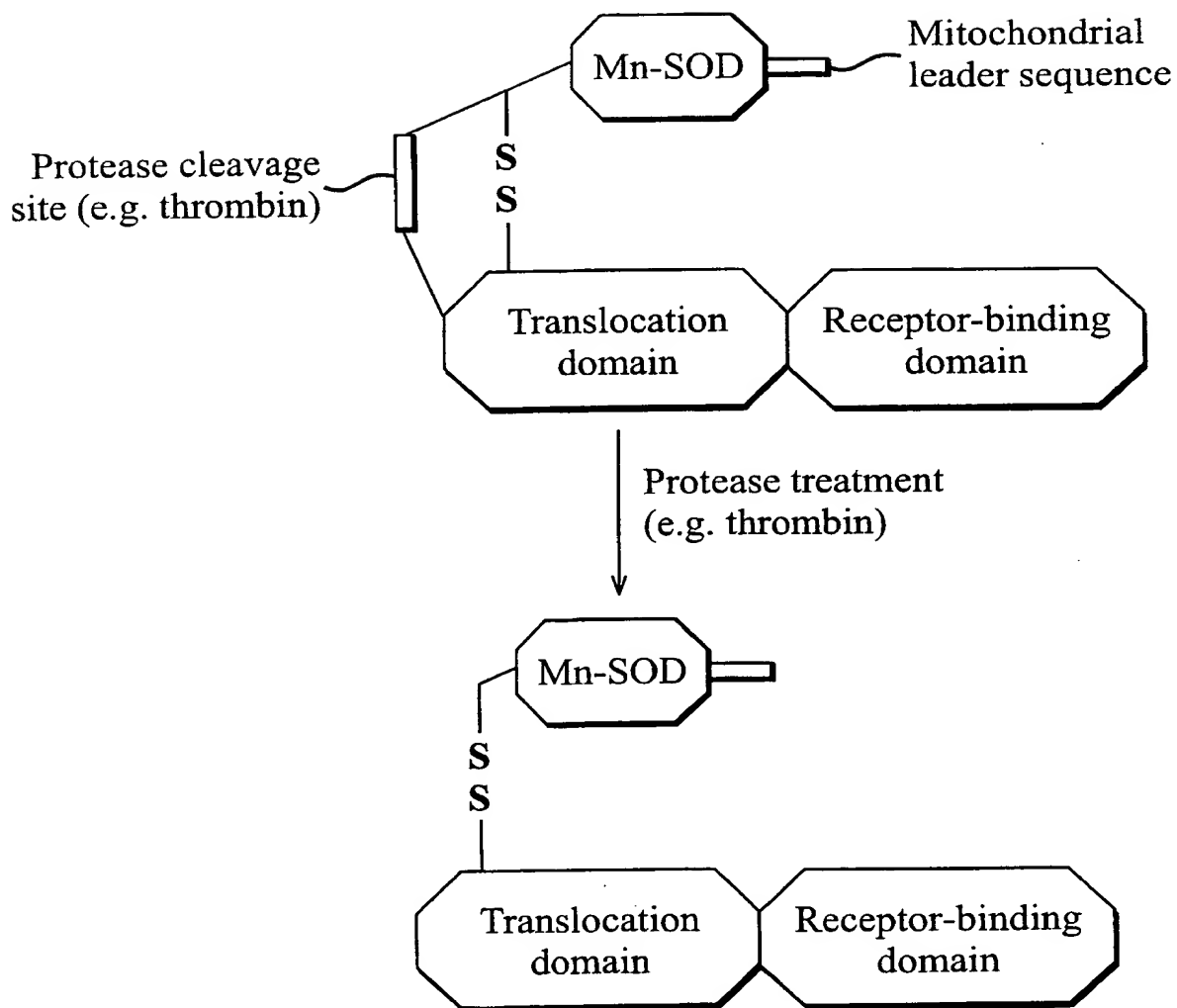


FIG. 2



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FIG. 3



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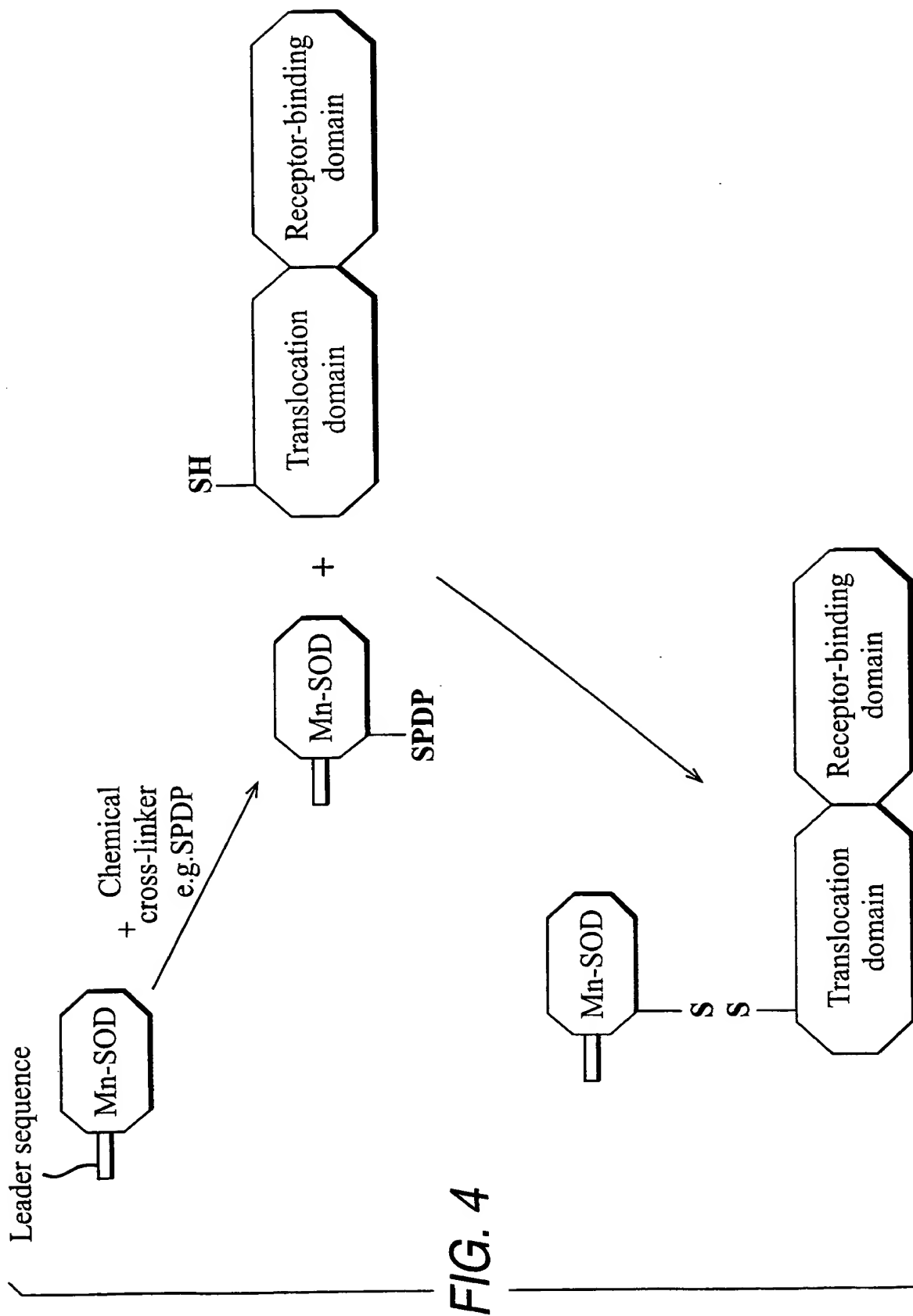
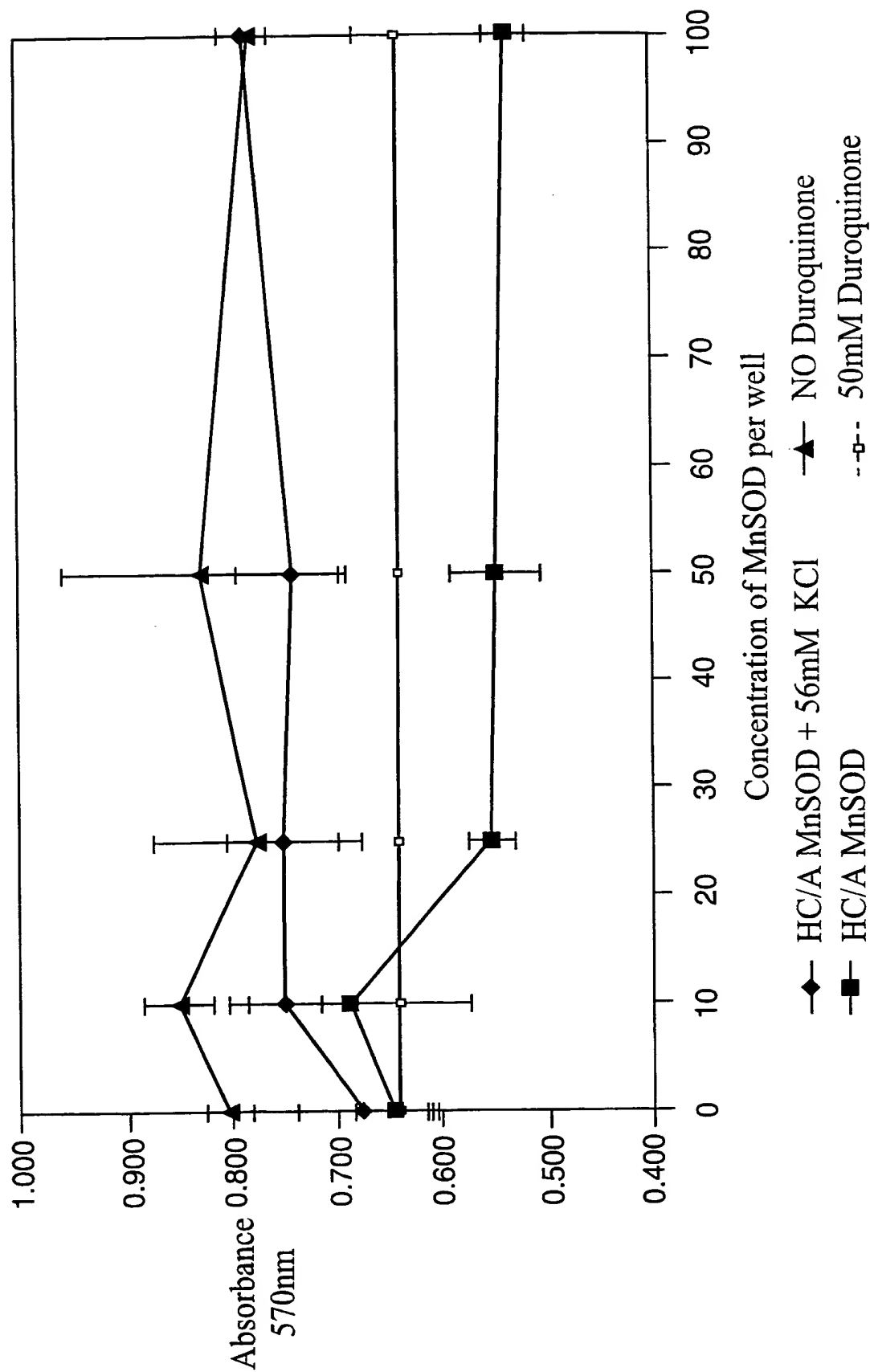


FIG. 4

4/4

FIG. 5 Demonstration of protective effects of HC/A Mnsod on NG108 cells subjected to oxidative stress by the addition of 50 μ M duroquinone for 4 hours.



- 1 -

SEQUENCE LISTING

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 HALLIS, Bassam
 SILMAN, Nigel
 SHONE, Clifford Charles
 SUTTON, John Mark

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 <212> PRT
 <213> Artificial Sequence

55 <220>
 <223> Description of Artificial Sequence:construct

60 <400> 3
 Met Pro Phe Glu Leu Pro Ala Leu Pro Tyr Pro Tyr Asp Ala Leu Glu
 1 5 10 15
 Pro His Ile Asp Lys Glu Thr Met Asn Ile His His Thr Lys His His
 20 25 30
 65 Asn Thr Tyr Val Thr Asn Leu Asn Ala Ala Leu Glu Gly His Pro Asp
 35 40 45

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	Leu	Gln	Asn	Lys	Ser	Leu	Glu	Glu	Leu	Leu	Ser	Asn	Leu	Glu	Ala	Leu
	50						55					60				
5	Pro	Glu	Ser	Ile	Arg	Thr	Ala	Val	Arg	Asn	Asn	Gly	Gly	Gly	His	Ala
	65					70				75						80
	Asn	His	Ser	Leu	Phe	Trp	Thr	Ile	Leu	Ser	Pro	Asn	Gly	Gly	Gly	Glu
					85					90					95	
10	Pro	Thr	Gly	Glu	Leu	Ala	Asp	Ala	Ile	Asn	Lys	Lys	Phe	Gly	Ser	Phe
				100					105					110		
	Thr	Ala	Phe	Lys	Asp	Glu	Phe	Ser	Lys	Ala	Ala	Ala	Gly	Arg	Phe	Gly
15			115					120					125			
	Ser	Gly	Trp	Ala	Trp	Leu	Val	Val	Asn	Asn	Gly	Glu	Leu	Glu	Ile	Thr
	130						135					140				
20	Ser	Thr	Pro	Asn	Gln	Asp	Ser	Pro	Ile	Met	Glu	Gly	Lys	Thr	Pro	Ile
	145					150					155					160
	Leu	Gly	Leu	Asp	Val	Trp	Glu	His	Ala	Tyr	Tyr	Leu	Lys	Tyr	Gln	Asn
				165						170					175	
25	Arg	Arg	Pro	Glu	Tyr	Ile	Ala	Ala	Phe	Trp	Asn	Val	Val	Asn	Trp	Asp
				180					185					190		
	Glu	Val	Ala	Lys	Arg	Tyr	Ser	Glu	Ala	Lys	Ala	Lys	Gln	Arg	Ser	Cys
30			195					200					205			
	Gly	Leu	Val	Pro	Arg	Gly	Ser	Gly	Pro	Gly	Ser	Ala	Leu	Asn	Asp	Leu
	210						215					220				
35	Cys	Ile	Lys	Val	Asn	Asn	Trp	Asp	Leu	Phe	Phe	Ser	Pro	Ser	Glu	Asp
	225					230					235					240
	Asn	Phe	Thr	Asn	Asp	Leu	Asn	Lys	Gly	Glu	Glu	Ile	Thr	Ser	Asp	Thr
				245						250					255	
40	Asn	Ile	Glu	Ala	Ala	Glu	Glu	Asn	Ile	Ser	Leu	Asp	Leu	Ile	Gln	Gln
				260					265					270		
	Tyr	Tyr	Leu	Thr	Phe	Asn	Phe	Asp	Asn	Glu	Pro	Glu	Asn	Ile	Ser	Ile
45			275					280					285			
	Glu	Asn	Leu	Ser	Ser	Asp	Ile	Ile	Gly	Gln	Leu	Glu	Leu	Met	Pro	Asn
	290						295					300				
50	Ile	Glu	Arg	Phe	Pro	Asn	Gly	Lys	Lys	Tyr	Glu	Leu	Asp	Lys	Tyr	Thr
	305					310					315					320
	Met	Phe	His	Tyr	Leu	Arg	Ala	Gln	Glu	Phe	Glu	His	Gly	Lys	Ser	Arg
					325					330					335	
55	Ile	Ala	Leu	Thr	Asn	Ser	Val	Asn	Glu	Ala	Leu	Leu	Asn	Pro	Ser	Arg
				340					345					350		
	Val	Tyr	Thr	Phe	Phe	Ser	Ser	Asp	Tyr	Val	Lys	Lys	Val	Asn	Lys	Ala
60			355					360					365			
	Thr	Glu	Ala	Ala	Met	Phe	Leu	Gly	Trp	Val	Glu	Gln	Leu	Val	Tyr	Asp
	370						375					380				
65	Phe	Thr	Asp	Glu	Thr	Ser	Glu	Val	Ser	Thr	Thr	Asp	Lys	Ile	Ala	Asp
	385					390					395					400
	Ile	Thr	Ile	Ile	Ile	Pro	Tyr	Ile	Gly	Pro	Ala	Leu	Asn	Ile	Gly	Asn

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	405								410				415			
5	Met	Leu	Tyr	Lys	Asp	Asp	Phe	Val	Gly	Ala	Leu	Ile	Phe	Ser	Gly	Ala
				420					425					430		
	Val	Ile	Leu	Leu	Glu	Phe	Ile	Pro	Glu	Ile	Ala	Ile	Pro	Val	Leu	Gly
			435					440					445			
10	Thr	Phe	Ala	Leu	Val	Ser	Tyr	Ile	Ala	Asn	Lys	Val	Leu	Thr	Val	Gln
		450					455					460				
	Thr	Ile	Asp	Asn	Ala	Leu	Ser	Lys	Arg	Asn	Glu	Lys	Trp	Asp	Glu	Val
	465					470					475				480	
15	Tyr	Lys	Tyr	Ile	Val	Thr	Asn	Trp	Leu	Ala	Lys	Val	Asn	Thr	Gln	Ile
					485					490					495	
	Asp	Leu	Ile	Arg	Lys	Lys	Met	Lys	Glu	Ala	Leu	Glu	Asn	Gln	Ala	Glu
20				500					505					510		
	Ala	Thr	Lys	Ala	Ile	Ile	Asn	Tyr	Gln	Tyr	Asn	Gln	Tyr	Thr	Glu	Glu
			515					520					525			
25	Glu	Lys	Asn	Asn	Ile	Asn	Phe	Asn	Ile	Asp	Asp	Leu	Ser	Ser	Lys	Leu
	530						535					540				
	Asn	Glu	Ser	Ile	Asn	Lys	Ala	Met	Ile	Asn	Ile	Asn	Lys	Phe	Leu	Asn
	545					550					555				560	
30	Gln	Cys	Ser	Val	Ser	Tyr	Leu	Met	Asn	Ser	Met	Ile	Pro	Tyr	Gly	Val
					565					570					575	
	Lys	Arg	Leu	Glu	Asp	Phe	Asp	Ala	Ser	Leu	Lys	Asp	Ala	Leu	Leu	Lys
35				580					585					590		
	Tyr	Ile	Tyr	Asp	Asn	Arg	Gly	Thr	Leu	Ile	Gly	Gln	Val	Asp	Arg	Leu
			595					600					605			
40	Lys	Asp	Lys	Val	Asn	Asn	Thr	Leu	Ser	Thr	Asp	Ile	Pro	Phe	Gln	Leu
	610						615					620				
	Ser	Lys	Tyr	Val	Asp	Asn	Gln	Arg	Leu	Leu	Ser	Thr	Phe	Thr	Glu	Tyr
	625					630					635				640	
45	Ile	Lys	Asn	Ile	Ile	Asn	Thr	Ser	Ile	Leu	Asn	Leu	Arg	Tyr	Glu	Ser
					645					650					655	
	Asn	His	Leu	Ile	Asp	Leu	Ser	Arg	Tyr	Ala	Ser	Lys	Ile	Asn	Ile	Gly
50				660					665					670		
	Ser	Lys	Val	Asn	Phe	Asp	Pro	Ile	Asp	Lys	Asn	Gln	Ile	Gln	Leu	Phe
			675					680					685			
55	Asn	Leu	Glu	Ser	Ser	Lys	Ile	Glu	Val	Ile	Leu	Lys	Asn	Ala	Ile	Val
	690						695					700				
	Tyr	Asn	Ser	Met	Tyr	Glu	Asn	Phe	Ser	Thr	Ser	Phe	Trp	Ile	Arg	Ile
	705					710					715				720	
60	Pro	Lys	Tyr	Phe	Asn	Ser	Ile	Ser	Leu	Asn	Asn	Glu	Tyr	Thr	Ile	Ile
					725					730					735	
	Asn	Cys	Met	Glu	Asn	Asn	Ser	Gly	Trp	Lys	Val	Ser	Leu	Asn	Tyr	Gly
				740					745					750		
65	Glu	Ile	Ile	Trp	Thr	Leu	Gln	Asp	Thr	Gln	Glu	Ile	Lys	Gln	Arg	Val
			755					760					765			

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	Val	Phe	Lys	Tyr	Ser	Gln	Met	Ile	Asn	Ile	Ser	Asp	Tyr	Ile	Asn	Arg
	770						775									
5	Trp	Ile	Phe	Val	Thr	Ile	Thr	Asn	Asn	Arg	Leu	Asn	Asn	Ser	Lys	Ile
	785					790					795					800
	Tyr	Ile	Asn	Gly	Arg	Leu	Ile	Asp	Gln	Lys	Pro	Ile	Ser	Asn	Leu	Gly
					805					810					815	
10	Asn	Ile	His	Ala	Ser	Asn	Asn	Ile	Met	Phe	Lys	Leu	Asp	Gly	Cys	Arg
				820					825					830		
	Asp	Thr	His	Arg	Tyr	Ile	Trp	Ile	Lys	Tyr	Phe	Asn	Leu	Phe	Asp	Lys
			835				840						845			
15	Glu	Leu	Asn	Glu	Lys	Glu	Ile	Lys	Asp	Leu	Tyr	Asp	Asn	Gln	Ser	Asn
	850					855						860				
20	Ser	Gly	Ile	Leu	Lys	Asp	Phe	Trp	Gly	Asp	Tyr	Leu	Gln	Tyr	Asp	Lys
	865					870					875					880
	Pro	Tyr	Tyr	Met	Leu	Asn	Leu	Tyr	Asp	Pro	Asn	Lys	Tyr	Val	Asp	Val
					885					890					895	
25	Asn	Asn	Val	Gly	Ile	Arg	Gly	Tyr	Met	Tyr	Leu	Lys	Gly	Pro	Arg	Gly
				900					905					910		
	Ser	Val	Met	Thr	Thr	Asn	Ile	Tyr	Leu	Asn	Ser	Ser	Leu	Tyr	Arg	Gly
			915					920					925			
30	Thr	Lys	Phe	Ile	Ile	Lys	Lys	Tyr	Ala	Ser	Gly	Asn	Lys	Asp	Asn	Ile
	930					935						940				
35	Val	Arg	Asn	Asn	Asp	Arg	Val	Tyr	Ile	Asn	Val	Val	Val	Lys	Asn	Lys
	945					950					955					960
	Glu	Tyr	Arg	Leu	Ala	Thr	Asn	Ala	Ser	Gln	Ala	Gly	Val	Glu	Lys	Ile
					965					970					975	
40	Leu	Ser	Ala	Leu	Glu	Ile	Pro	Asp	Val	Gly	Asn	Leu	Ser	Gln	Val	Val
				980					985					990		
	Val	Met	Lys	Ser	Lys	Asn	Asp	Gln	Gly	Ile	Thr	Asn	Lys	Cys	Lys	Met
			995				1000						1005			
45	Asn	Leu	Gln	Asp	Asn	Asn	Gly	Asn	Asp	Ile	Gly	Phe	Ile	Gly	Phe	His
	1010						1015					1020				
50	Gln	Phe	Asn	Asn	Ile	Ala	Lys	Leu	Val	Ala	Ser	Asn	Trp	Tyr	Asn	Arg
	1025					1030					1035					1040
	Gln	Ile	Glu	Arg	Ser	Ser	Arg	Thr								

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	Met	Pro	Phe	Glu	Leu	Pro	Ala	Leu	Pro	Tyr	Pro	Tyr	Asp	Ala	Leu	Glu
	1				5					10					15	
5	Pro	His	Ile	Asp	Lys	Glu	Thr	Met	Asn	Ile	His	His	Thr	Lys	His	His
				20					25					30		
	Asn	Thr	Tyr	Val	Thr	Asn	Leu	Asn	Ala	Ala	Leu	Glu	Gly	His	Pro	Asp
10			35					40					45			
	Leu	Gln	Asn	Lys	Ser	Leu	Glu	Leu	Leu	Ser	Asn	Leu	Glu	Ala	Leu	
	50						55				60					
15	Pro	Glu	Ser	Ile	Arg	Thr	Ala	Val	Arg	Asn	Asn	Gly	Gly	Gly	His	Ala
	65					70					75					80
	Asn	His	Ser	Leu	Phe	Trp	Thr	Ile	Leu	Ser	Pro	Asn	Gly	Gly	Gly	Glu
					85					90					95	
20	Pro	Thr	Gly	Glu	Leu	Ala	Asp	Ala	Ile	Asn	Lys	Lys	Phe	Gly	Ser	Phe
				100					105					110		
	Thr	Ala	Phe	Lys	Asp	Glu	Phe	Ser	Lys	Ala	Ala	Ala	Gly	Arg	Phe	Gly
25			115					120					125			
	Ser	Gly	Trp	Ala	Trp	Leu	Val	Val	Asn	Asn	Gly	Glu	Leu	Glu	Ile	Thr
	130						135					140				
30	Ser	Thr	Pro	Asn	Gln	Asp	Ser	Pro	Ile	Met	Glu	Gly	Lys	Thr	Pro	Ile
	145					150					155					160
	Leu	Gly	Leu	Asp	Val	Trp	Glu	His	Ala	Tyr	Tyr	Leu	Lys	Tyr	Gln	Asn
					165					170					175	
35	Arg	Arg	Pro	Glu	Tyr	Ile	Ala	Ala	Phe	Trp	Asn	Val	Val	Asn	Trp	Asp
				180					185					190		
	Glu	Val	Ala	Lys	Arg	Tyr	Ser	Glu	Ala	Lys	Ala	Lys	Gln	Arg	Ser	Cys
40			195					200					205			
	Gly	Leu	Val	Pro	Arg	Gly	Ser	Gly	Pro	Gly	Ser	Lys	Ala	Pro	Gly	Ile
	210						215					220				
45	Cys	Ile	Asp	Val	Asp	Asn	Glu	Asp	Leu	Phe	Phe	Ile	Ala	Asp	Lys	Asn
	225					230					235					240
	Ser	Phe	Ser	Asp	Asp	Leu	Ser	Lys	Asn	Glu	Arg	Ile	Glu	Tyr	Asn	Thr
					245					250					255	
50	Gln	Ser	Asn	Tyr	Ile	Glu	Asn	Asp	Phe	Pro	Ile	Asn	Glu	Leu	Ile	Leu
				260					265					270		
	Asp	Thr	Asp	Leu	Ile	Ser	Lys	Ile	Glu	Leu	Pro	Ser	Glu	Asn	Thr	Glu
55			275					280					285			
	Ser	Leu	Thr	Asp	Phe	Asn	Val	Asp	Val	Pro	Val	Tyr	Glu	Lys	Gln	Pro
	290						295					300				
60	Ala	Ile	Lys	Lys	Ile	Phe	Thr	Asp	Glu	Asn	Thr	Ile	Phe	Gln	Tyr	Leu
	305					310					315					320
	Tyr	Ser	Gln	Thr	Phe	Pro	Leu	Asp	Ile	Arg	Asp	Ile	Ser	Leu	Thr	Ser
					325					330					335	
65	Ser	Phe	Asp	Asp	Ala	Leu	Leu	Phe	Ser	Asn	Lys	Val	Tyr	Ser	Phe	Phe
				340					345					350		

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	Ser	Met	Asp	Tyr	Ile	Lys	Thr	Ala	Asn	Lys	Val	Val	Glu	Ala	Gly	Leu
			355					360					365			
5	Phe	Ala	Gly	Trp	Val	Lys	Gln	Ile	Val	Asn	Asp	Phe	Val	Ile	Glu	Ala
		370					375					380				
	Asn	Lys	Ser	Asn	Thr	Met	Asp	Lys	Ile	Ala	Asp	Ile	Ser	Leu	Ile	Val
	385					390					395					400
10	Pro	Tyr	Ile	Gly	Leu	Ala	Leu	Asn	Val	Gly	Asn	Glu	Thr	Ala	Lys	Gly
				405						410					415	
	Asn	Phe	Glu	Asn	Ala	Phe	Glu	Ile	Ala	Gly	Ala	Ser	Ile	Leu	Leu	Glu
15				420					425					430		
	Phe	Ile	Pro	Glu	Leu	Leu	Ile	Pro	Val	Val	Gly	Ala	Phe	Leu	Leu	Glu
			435					440					445			
20	Ser	Tyr	Ile	Asp	Asn	Lys	Asn	Lys	Ile	Ile	Lys	Thr	Ile	Asp	Asn	Ala
	450						455					460				
	Leu	Thr	Lys	Arg	Asn	Glu	Lys	Trp	Ser	Asp	Met	Tyr	Gly	Leu	Ile	Val
	465					470					475					480
25	Ala	Gln	Trp	Leu	Ser	Thr	Val	Asn	Thr	Gln	Phe	Tyr	Thr	Ile	Lys	Glu
				485						490					495	
	Gly	Met	Tyr	Lys	Ala	Leu	Asn	Tyr	Gln	Ala	Gln	Ala	Leu	Glu	Glu	Ile
30				500					505					510		
	Ile	Lys	Tyr	Arg	Tyr	Asn	Ile	Tyr	Ser	Glu	Lys	Glu	Lys	Ser	Asn	Ile
			515					520					525			
35	Asn	Ile	Asp	Phe	Asn	Asp	Ile	Asn	Ser	Lys	Leu	Asn	Glu	Gly	Ile	Asn
	530						535					540				
	Gln	Ala	Ile	Asp	Asn	Ile	Asn	Asn	Phe	Ile	Asn	Gly	Cys	Ser	Val	Ser
	545					550					555					560
40	Tyr	Leu	Met	Lys	Lys	Met	Ile	Pro	Leu	Ala	Val	Glu	Lys	Leu	Leu	Asp
				565						570					575	
	Phe	Asp	Asn	Thr	Leu	Lys	Lys	Asn	Leu	Leu	Asn	Tyr	Ile	Asp	Glu	Asn
45				580					585					590		
	Lys	Leu	Tyr	Leu	Ile	Gly	Ser	Ala	Glu	Tyr	Glu	Lys	Ser	Lys	Val	Asn
			595					600					605			
50	Lys	Tyr	Leu	Lys	Thr	Ile	Met	Pro	Phe	Asp	Leu	Ser	Ile	Tyr	Thr	Asn
	610						615					620				
	Asp	Thr	Ile	Leu	Ile	Glu	Met	Phe	Asn	Lys	Tyr	Asn	Ser	Glu	Ile	Leu
	625					630					635					640
55	Asn	Asn	Ile	Ile	Leu	Asn	Leu	Arg	Tyr	Lys	Asp	Asn	Asn	Leu	Ile	Asp
					645					650					655	
	Leu	Ser	Gly	Tyr	Gly	Ala	Lys	Val	Glu	Val	Tyr	Asp	Gly	Val	Glu	Leu
60				660					665					670		
	Asn	Asp	Lys	Asn	Gln	Phe	Lys	Leu	Thr	Ser	Ser	Ala	Asn	Ser	Lys	Ile
			675					680					685			
65	Arg	Val	Thr	Gln	Asn	Gln	Asn	Ile	Ile	Phe	Asn	Ser	Val	Phe	Leu	Asp
	690						695					700				
	Phe	Ser	Val	Ser	Phe	Trp	Ile	Arg	Ile	Pro	Lys	Tyr	Lys	Asn	Asp	Gly

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	705		710		715		720
	Ile Gln Asn Tyr	Ile His Asn Glu Tyr Thr	Ile Ile Asn Cys Met Lys				
		725	730			735	
5	Asn Asn Ser Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp						
		740	745			750	
10	Thr Leu Ile Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr						
		755	760			765	
	Asn Ile Arg Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val						
		770	775			780	
15	Thr Ile Thr Asn Asn Leu Asn Asn Ala Lys Ile Tyr Ile Asn Gly Lys						
		785	790			795	800
	Leu Glu Ser Asn Thr Asp Ile Lys Asp Ile Arg Glu Val Ile Ala Asn						
20		805	810			815	
	Gly Glu Ile Ile Phe Lys Leu Asp Gly Asp Ile Asp Arg Thr Gln Phe						
		820	825			830	
25	Ile Trp Met Lys Tyr Phe Ser Ile Phe Asn Thr Glu Leu Ser Gln Ser						
		835	840			845	
	Asn Ile Glu Glu Arg Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys						
		850	855			860	
30	Asp Phe Trp Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe						
		865	870			875	880
	Asn Ala Gly Asn Lys Asn Ser Tyr Ile Lys Leu Lys Lys Asp Ser Pro						
35		885	890			895	
	Val Gly Glu Ile Leu Thr Arg Ser Lys Tyr Asn Gln Asn Ser Lys Tyr						
		900	905			910	
40	Ile Asn Tyr Arg Asp Leu Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg						
		915	920			925	
	Lys Ser Asn Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp						
		930	935			940	
45	Tyr Ile Tyr Leu Asp Phe Phe Asn Leu Asn Gln Glu Trp Arg Val Tyr						
		945	950			955	960
	Thr Tyr Lys Tyr Phe Lys Lys Glu Glu Glu Lys Leu Phe Leu Ala Pro						
50		965	970			975	
	Ile Ser Asp Ser Asp Glu Phe Tyr Asn Thr Ile Gln Ile Lys Glu Tyr						
		980	985			990	
55	Asp Glu Gln Pro Thr Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu						
		995	1000			1005	
	Glu Ser Thr Asp Glu Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu						
		1010	1015			1020	
60	Ser Gly Ile Val Phe Glu Glu Tyr Lys Asp Tyr Phe Cys Ile Ser Lys						
		1025	1030			1035	1040
	Trp Tyr Leu Lys Glu Val Lys Arg Lys Pro Tyr Asn Leu Lys Leu Gly						
		1045	1050			1055	
65	Cys Asn Trp Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu						
		1060	1065			1070	

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5 <210> 5
 <211> 1059
 <212> PRT
 <213> Artificial Sequence

10 <220>
 <223> Description of Artificial Sequence:construct

15 <400> 5
 Met Pro Phe Glu Leu Pro Ala Leu Pro Tyr Pro Tyr Asp Ala Leu Glu
 1 5 10 15
 Pro His Ile Asp Lys Glu Thr Met Asn Ile His His Thr Lys His His
 20 25 30
 Asn Thr Tyr Val Thr Asn Leu Asn Ala Ala Leu Glu Gly His Pro Asp
 35 40 45
 Leu Gln Asn Lys Ser Leu Glu Glu Leu Leu Ser Asn Leu Glu Ala Leu
 50 55 60
 Pro Glu Ser Ile Arg Thr Ala Val Arg Asn Asn Gly Gly Gly His Ala
 65 70 75 80
 Asn His Ser Leu Phe Trp Thr Ile Leu Ser Pro Asn Gly Gly Gly Glu
 85 90 95
 Pro Thr Gly Glu Leu Ala Asp Ala Ile Asn Lys Lys Phe Gly Ser Phe
 100 105 110
 Thr Ala Phe Lys Asp Glu Phe Ser Lys Ala Ala Ala Gly Arg Phe Gly
 115 120 125
 Ser Gly Trp Ala Trp Leu Val Val Asn Asn Gly Glu Leu Glu Ile Thr
 130 135 140
 Ser Thr Pro Asn Gln Asp Ser Pro Ile Met Glu Gly Lys Thr Pro Ile
 145 150 155 160
 Leu Gly Leu Asp Val Trp Glu His Ala Tyr Tyr Leu Lys Tyr Gln Asn
 165 170 175
 Arg Arg Pro Glu Tyr Ile Ala Ala Phe Trp Asn Val Val Asn Trp Asp
 180 185 190
 Glu Val Ala Lys Arg Tyr Ser Glu Ala Lys Ala Lys Gln Arg Ser Cys
 195 200 205
 Gly Leu Val Pro Arg Gly Ser Gly Pro Gly Ser Lys Ala Pro Pro Arg
 210 215 220
 Leu Cys Ile Arg Val Asn Asn Arg Glu Leu Phe Phe Val Ala Ser Glu
 225 230 235 240
 Ser Ser Tyr Asn Glu Asn Asp Ile Asn Thr Pro Lys Glu Ile Asp Asp
 245 250 255
 Thr Thr Asn Leu Asn Asn Asn Tyr Arg Asn Asn Leu Asp Glu Val Ile
 260 265 270
 Leu Asp Tyr Asn Ser Glu Thr Ile Pro Gln Ile Ser Asn Gln Thr Leu
 275 280 285
 sn Thr Leu Val Gln Asp Asp Ser Tyr Val Pro Arg Tyr Asp Ser Asn
 290 295 300

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	Gly	Thr	Ser	Glu	Ile	Glu	Glu	His	Asn	Val	Val	Asp	Leu	Asn	Val	Phe
	305					310					315					320
5	Phe	Tyr	Leu	His	Ala	Gln	Lys	Val	Pro	Glu	Gly	Glu	Thr	Asn	Ile	Ser
					325					330					335	
	Leu	Thr	Ser	Ser	Ile	Asp	Thr	Ala	Leu	Ser	Glu	Glu	Ser	Gln	Val	Tyr
				340					345					350		
10	Thr	Phe	Phe	Ser	Ser	Glu	Phe	Ile	Asn	Thr	Ile	Asn	Lys	Pro	Val	His
			355					360					365			
	Ala	Ala	Leu	Phe	Ile	Ser	Trp	Ile	Asn	Gln	Val	Ile	Arg	Asp	Phe	Thr
15		370					375					380				
	Thr	Glu	Ala	Thr	Gln	Lys	Ser	Thr	Phe	Asp	Lys	Ile	Ala	Asp	Ile	Ser
	385					390					395					400
20	Leu	Val	Val	Pro	Tyr	Val	Gly	Leu	Ala	Leu	Asn	Ile	Gly	Asn	Glu	Val
					405					410					415	
	Gln	Lys	Glu	Asn	Phe	Lys	Glu	Ala	Phe	Glu	Leu	Leu	Gly	Ala	Gly	Ile
				420					425					430		
25	Leu	Leu	Glu	Phe	Val	Pro	Glu	Leu	Leu	Ile	Pro	Thr	Ile	Leu	Val	Phe
			435					440					445			
	Thr	Ile	Lys	Ser	Phe	Ile	Gly	Ser	Ser	Glu	Asn	Lys	Asn	Lys	Ile	Ile
30		450					455					460				
	Lys	Ala	Ile	Asn	Asn	Ser	Leu	Met	Glu	Arg	Glu	Thr	Lys	Trp	Lys	Glu
	465					470					475					480
35	Ile	Tyr	Ser	Trp	Ile	Val	Ser	Asn	Trp	Leu	Thr	Arg	Ile	Asn	Thr	Gln
				485						490					495	
	Phe	Asn	Lys	Arg	Lys	Glu	Gln	Met	Tyr	Gln	Ala	Leu	Gln	Asn	Gln	Val
				500					505					510		
40	Asp	Ala	Ile	Lys	Thr	Val	Ile	Glu	Tyr	Lys	Tyr	Asn	Asn	Tyr	Thr	Ser
			515					520					525			
	Asp	Glu	Arg	Asn	Arg	Leu	Glu	Ser	Glu	Tyr	Asn	Ile	Asn	Asn	Ile	Arg
45		530					535					540				
	Glu	Glu	Leu	Asn	Lys	Lys	Val	Ser	Leu	Ala	Met	Glu	Asn	Ile	Glu	Arg
	545					550					555					560
50	Phe	Ile	Thr	Glu	Ser	Ser	Ile	Phe	Tyr	Leu	Met	Lys	Leu	Ile	Asn	Glu
					565					570					575	
	Ala	Lys	Val	Ser	Lys	Leu	Arg	Glu	Tyr	Asp	Glu	Gly	Val	Lys	Glu	Tyr
				580					585					590		
55	Leu	Leu	Asp	Tyr	Ile	Ser	Glu	His	Arg	Ser	Ile	Leu	Gly	Asn	Ser	Val
			595					600					605			
	Gln	Glu	Leu	Asn	Asp	Leu	Val	Thr	Ser	Thr	Leu	Asn	Asn	Ser	Ile	Pro
60		610					615					620				
	Phe	Glu	Leu	Ser	Ser	Tyr	Thr	Asn	Asp	Lys	Ile	Leu	Ile	Leu	Tyr	Phe
	625					630					635					640
65	Asn	Lys	Leu	Tyr	Lys	Lys	Ile	Lys	Asp	Asn	Ser	Ile	Leu	Asp	Met	Arg
					645					650					655	
	Tyr	Glu	Asn	Asn	Lys	Phe	Ile	Asp	Ile	Ser	Gly	Tyr	Gly	Ser	Asn	Ile

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	660	665	670
5	Ser Ile Asn Gly Asp Val Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe 675 680 685		
	Gly Ile Tyr Ser Ser Lys Pro Ser Glu Val Asn Ile Ala Gln Asn Asn 690 695 700		
10	Asp Ile Ile Tyr Asn Gly Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp 705 710 715 720		
	Val Arg Ile Pro Lys Tyr Phe Asn Lys Val Asn Leu Asn Asn Glu Tyr 725 730 735		
15	Thr Ile Ile Asp Cys Ile Arg Asn Asn Asn Ser Gly Trp Lys Ile Ser 740 745 750		
	Leu Asn Tyr Asn Lys Ile Ile Trp Thr Leu Gln Asp Thr Ala Gly Asn 755 760 765		
20	Asn Gln Lys Leu Val Phe Asn Tyr Thr Gln Met Ile Ser Ile Ser Asp 770 775 780		
	Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Gly 785 790 795 800		
25	Asn Ser Arg Ile Tyr Ile Asn Gly Asn Leu Ile Asp Glu Lys Ser Ile 805 810 815		
30	Ser Asn Leu Gly Asp Ile His Val Ser Asp Asn Ile Leu Phe Lys Ile 820 825 830		
	Val Gly Cys Asn Asp Thr Arg Tyr Val Gly Ile Arg Tyr Phe Lys Val 835 840 845		
35	Phe Asp Thr Glu Leu Gly Lys Thr Glu Ile Glu Thr Leu Tyr Ser Asp 850 855 860		
	Glu Pro Asp Pro Ser Ile Leu Lys Asp Phe Trp Gly Asn Tyr Leu Leu 865 870 875 880		
40	Tyr Asn Lys Arg Tyr Tyr Leu Leu Asn Leu Leu Arg Thr Asp Lys Ser 885 890 895		
45	Ile Thr Gln Asn Ser Asn Phe Leu Asn Ile Asn Gln Gln Arg Gly Val 900 905 910		
	Tyr Gln Lys Pro Asn Ile Phe Ser Asn Thr Arg Leu Tyr Thr Gly Val 915 920 925		
50	Glu Val Ile Ile Arg Lys Asn Gly Ser Thr Asp Ile Ser Asn Thr Asp 930 935 940		
	Asn Phe Val Arg Lys Asn Asp Leu Ala Tyr Ile Asn Val Val Asp Arg 945 950 955 960		
55	Asp Val Glu Tyr Arg Leu Tyr Ala Asp Ile Ser Ile Ala Lys Pro Glu 965 970 975		
60	Lys Ile Ile Lys Leu Ile Arg Thr Ser Asn Ser Asn Asn Ser Leu Gly 980 985 990		
	Gln Ile Ile Val Met Asp Ser Ile Gly Asn Asn Cys Thr Met Asn Phe 995 1000 1005		
65	Gln Asn Asn Asn Gly Gly Asn Ile Gly Leu Leu Gly Phe His Ser Asn 1010 1015 1020		

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Asn Leu Val Ala Ser Ser Trp Tyr Tyr Asn Asn Ile Arg Lys Asn Thr
 1025 1030 1035 1040
 Ser Ser Asn Gly Cys Phe Trp Ser Phe Ile Ser Lys Glu His Gly Trp
 1045 1050 1055
 Gln Glu Asn
 <210> 6
 <211> 1092
 <212> PRT
 <213> Artificial Sequence
 <220>
 <223> Description of Artificial Sequence:construct
 <400> 6
 Met Leu Ser Arg Ala Val Cys Gly Thr Ser Arg Gln Leu Ala Pro Ala
 1 5 10 15
 Leu Gly Tyr Leu Gly Ser Arg Gln Lys His Ser Arg Gly Ser Pro Ala
 20 25 30
 Leu Pro Tyr Pro Tyr Asp Ala Leu Glu Pro His Ile Asp Lys Glu Thr
 35 40 45
 Met Asn Ile His His Thr Lys His His Asn Thr Tyr Val Thr Asn Leu
 50 55 60
 Asn Ala Ala Leu Glu Gly His Pro Asp Leu Gln Asn Lys Ser Leu Glu
 65 70 75 80
 Glu Leu Leu Ser Asn Leu Glu Ala Leu Pro Glu Ser Ile Arg Thr Ala
 85 90 95
 Val Arg Asn Asn Gly Gly Gly His Ala Asn His Ser Leu Phe Trp Thr
 100 105 110
 Ile Leu Ser Pro Asn Gly Gly Gly Glu Pro Thr Gly Glu Leu Ala Asp
 115 120 125
 Ala Ile Asn Lys Lys Phe Gly Ser Phe Thr Ala Phe Lys Asp Glu Phe
 130 135 140
 Ser Lys Ala Ala Ala Gly Arg Phe Gly Ser Gly Trp Ala Trp Leu Val
 145 150 155 160
 Val Asn Asn Gly Glu Leu Glu Ile Thr Ser Thr Pro Asn Gln Asp Ser
 165 170 175
 Pro Ile Met Glu Gly Lys Thr Pro Ile Leu Gly Leu Asp Val Trp Glu
 180 185 190
 His Ala Tyr Tyr Leu Lys Tyr Gln Asn Arg Arg Pro Glu Tyr Ile Ala
 195 200 205
 Ala Phe Trp Asn Val Val Asn Trp Asp Glu Val Ala Lys Arg Tyr Ser
 210 215 220
 Glu Ala Lys Ala Lys Gln Arg Ser Cys Gly Leu Val Pro Arg Gly Ser
 225 230 235 240
 Gly Pro Gly Ser Ala Leu Asn Asp Leu Cys Ile Lys Val Asn Asn Trp
 245 250 255

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	Asp	Leu	Phe	Phe	Ser	Pro	Ser	Glu	Asp	Asn	Phe	Thr	Asn	Asp	Leu	Asn
				260					265					270		
5	Lys	Gly	Glu	Glu	Ile	Thr	Ser	Asp	Thr	Asn	Ile	Glu	Ala	Ala	Glu	Glu
			275					280					285			
	Asn	Ile	Ser	Leu	Asp	Leu	Ile	Gln	Gln	Tyr	Tyr	Leu	Thr	Phe	Asn	Phe
	290						295					300				
10	Asp	Asn	Glu	Pro	Glu	Asn	Ile	Ser	Ile	Glu	Asn	Leu	Ser	Ser	Asp	Ile
	305					310					315					320
	Ile	Gly	Gln	Leu	Glu	Leu	Met	Pro	Asn	Ile	Glu	Arg	Phe	Pro	Asn	Gly
15					325					330					335	
	Lys	Lys	Tyr	Glu	Leu	Asp	Lys	Tyr	Thr	Met	Phe	His	Tyr	Leu	Arg	Ala
				340					345					350		
20	Gln	Glu	Phe	Glu	His	Gly	Lys	Ser	Arg	Ile	Ala	Leu	Thr	Asn	Ser	Val
			355					360					365			
	Asn	Glu	Ala	Leu	Leu	Asn	Pro	Ser	Arg	Val	Tyr	Thr	Phe	Phe	Ser	Ser
	370						375					380				
25	Asp	Tyr	Val	Lys	Lys	Val	Asn	Lys	Ala	Thr	Glu	Ala	Ala	Met	Phe	Leu
	385					390					395					400
	Gly	Trp	Val	Glu	Gln	Leu	Val	Tyr	Asp	Phe	Thr	Asp	Glu	Thr	Ser	Glu
30					405					410					415	
	Val	Ser	Thr	Thr	Asp	Lys	Ile	Ala	Asp	Ile	Thr	Ile	Ile	Ile	Pro	Tyr
				420					425					430		
35	Ile	Gly	Pro	Ala	Leu	Asn	Ile	Gly	Asn	Met	Leu	Tyr	Lys	Asp	Asp	Phe
			435					440					445			
	Val	Gly	Ala	Leu	Ile	Phe	Ser	Gly	Ala	Val	Ile	Leu	Leu	Glu	Phe	Ile
		450					455					460				
40	Pro	Glu	Ile	Ala	Ile	Pro	Val	Leu	Gly	Thr	Phe	Ala	Leu	Val	Ser	Tyr
	465					470					475					480
	Ile	Ala	Asn	Lys	Val	Leu	Thr	Val	Gln	Thr	Ile	Asp	Asn	Ala	Leu	Ser
45					485					490					495	
	Lys	Arg	Asn	Glu	Lys	Trp	Asp	Glu	Val	Tyr	Lys	Tyr	Ile	Val	Thr	Asn
				500					505					510		
50	Trp	Leu	Ala	Lys	Val	Asn	Thr	Gln	Ile	Asp	Leu	Ile	Arg	Lys	Lys	Met
			515					520					525			
	Lys	Glu	Ala	Leu	Glu	Asn	Gln	Ala	Glu	Ala	Thr	Lys	Ala	Ile	Ile	Asn
		530					535					540				
55	Tyr	Gln	Tyr	Asn	Gln	Tyr	Thr	Glu	Glu	Glu	Lys	Asn	Asn	Ile	Asn	Phe
	545					550					555					560
	Asn	Ile	Asp	Asp	Leu	Ser	Ser	Lys	Leu	Asn	Glu	Ser	Ile	Asn	Lys	Ala
60					565					570					575	
	Met	Ile	Asn	Ile	Asn	Lys	Phe	Leu	Asn	Gln	Cys	Ser	Val	Ser	Tyr	Leu
				580					585					590		
65	Met	Asn	Ser	Met	Ile	Pro	Tyr	Gly	Val	Lys	Arg	Leu	Glu	Asp	Phe	Asp
			595					600					605			
	Ala	Ser	Leu	Lys	Asp	Ala	Leu	Leu	Lys	Tyr	Ile	Tyr	Asp	Asn	Arg	Gly

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	610	615	620	
5	Thr 625	Leu Ile Gly Gln Val 630	Asp Arg Leu Lys Asp 635	Lys Val Asn Asn Thr 640
10	Leu Ser Thr Asp 645	Ile Pro Phe Gln Leu 650	Lys Tyr Val Asp 655	Asn Gln
15	Arg Leu Leu Ser 660	Thr Phe Thr Glu Tyr Ile 665	Lys Asn Ile Ile 670	Asn Thr
20	Ser Ile 675	Leu Asn Leu Arg Tyr 680	Glu Ser Asn His Leu 685	Ile Asp Leu Ser
25	Arg Tyr 690	Ala Ser Lys Ile Asn 695	Ile Gly Ser Lys Val 700	Asn Phe Asp Pro
30	Ile 705	Asp Lys Asn Gln Ile 710	Gln Leu Phe Asn Leu 715	Glu Ser Ser Lys Ile 720
35	Glu Val Ile Leu 725	Lys Asn Ala Ile Val 730	Tyr Asn Ser Met Tyr 735	Glu Asn
40	Phe Ser Thr Ser 740	Phe Trp Ile Arg Ile 745	Pro Lys Tyr Phe 750	Asn Ser Ile
45	Ser Leu Asn Asn 755	Glu Tyr Thr Ile 760	Ile Asn Cys Met 765	Glu Asn Asn Ser
50	Gly Trp Lys Val Ser Leu 770	Asn Tyr Gly Glu Ile 775	Ile Ile Trp Thr Leu 780	Gln
55	Asp Thr Gln Glu Ile Lys 785	Gln Arg Val Val Phe 790	Lys Tyr Ser Gln Met 795	800
60	Ile Asn Ile Ser 805	Asp Tyr Ile Asn Arg Trp 810	Ile Phe Val Thr 815	Ile Thr
65	Asn Asn Arg Leu Asn Asn Ser Lys 820	Ile Tyr Ile Asn Gly Arg 825	Ile Asn Gly Arg 830	Leu Ile
70	Asp Gln Lys Pro Ile Ser Asn 835	Leu Gly Asn Ile His 840	Ala Ser Asn Asn 845	
75	Ile Met Phe Lys Leu Asp Gly 850	Cys Arg Asp Thr His 855	Arg Tyr Ile Trp 860	
80	Ile Lys Tyr Phe Asn Leu Phe Asp Lys Glu 865	Leu 870	Asn Glu Lys Glu 875	Ile 880
85	Lys Asp Leu Tyr Asp 885	Asn Gln Ser Asn Ser 890	Gly Ile Leu Lys Asp 895	Phe
90	Trp Gly Asp Tyr 900	Leu Gln Tyr Asp Lys 905	Pro Tyr Tyr Met Leu 910	Asn Leu
95	Tyr Asp Pro Asn Lys Tyr Val 915	Asp Val Asn Asn Val 920	Gly Ile Arg Gly 925	
100	Tyr Met Tyr Leu Lys Gly 930	Pro Arg Gly Ser Val 935	Met Thr Thr Asn Ile 940	
105	Tyr Leu Asn Ser Ser 945	Leu 950	Tyr Arg Gly Thr Lys 955	Phe Ile Ile Lys 960
110	Tyr Ala Ser Gly Asn 965	Lys Asp Asn Ile Val 970	Arg Asn Asn Asp Arg 975	Val

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Tyr Ile Asn Val Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn
 980 985 990
 5 Ala Ser Gln Ala Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro
 995 1000 1005
 Asp Val Gly Asn Leu Ser Gln Val Val Val Met Lys Ser Lys Asn Asp
 1010 1015 1020
 10 Gln Gly Ile Thr Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly
 1025 1030 1035 1040
 Asn Asp Ile Gly Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys
 1045 1050 1055
 15 Leu Val Ala Ser Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg
 1060 1065 1070
 20 Thr Leu Gly Cys Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly
 1075 1080 1085
 Glu Arg Pro Leu
 1090
 25
 <210> 7
 <211> 1095
 <212> PRT
 30 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence:construct
 35 <400> 7
 Met Leu Ser Arg Ala Val Cys Gly Thr Ser Arg Gln Leu Ala Pro Ala
 1 5 10 15
 40 Leu Gly Tyr Leu Gly Ser Arg Gln Lys His Ser Arg Gly Ser Pro Ala
 20 25 30
 Leu Pro Tyr Pro Tyr Asp Ala Leu Glu Pro His Ile Asp Lys Glu Thr
 35 40 45
 45 Met Asn Ile His His Thr Lys His His Asn Thr Tyr Val Thr Asn Leu
 50 55 60
 Asn Ala Ala Leu Glu Gly His Pro Asp Leu Gln Asn Lys Ser Leu Glu
 65 70 75 80
 50 Glu Leu Leu Ser Asn Leu Glu Ala Leu Pro Glu Ser Ile Arg Thr Ala
 85 90 95
 55 Val Arg Asn Asn Gly Gly Gly His Ala Asn His Ser Leu Phe Trp Thr
 100 105 110
 Ile Leu Ser Pro Asn Gly Gly Gly Glu Pro Thr Gly Glu Leu Ala Asp
 115 120 125
 60 Ala Ile Asn Lys Lys Phe Gly Ser Phe Thr Ala Phe Lys Asp Glu Phe
 130 135 140
 Ser Lys Ala Ala Ala Gly Arg Phe Gly Ser Gly Trp Ala Trp Leu Val
 145 150 155 160
 65 Val Asn Asn Gly Glu Leu Glu Ile Thr Ser Thr Pro Asn Gln Asp Ser
 165 170 175

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	Pro	Ile	Met	Glu	Gly	Lys	Thr	Pro	Ile	Leu	Gly	Leu	Asp	Val	Trp	Glu
				180					185					190		
5	His	Ala	Tyr	Tyr	Leu	Lys	Tyr	Gln	Asn	Arg	Arg	Pro	Glu	Tyr	Ile	Ala
			195					200					205			
	Ala	Phe	Trp	Asn	Val	Val	Asn	Trp	Asp	Glu	Val	Ala	Lys	Arg	Tyr	Ser
		210					215					220				
10	Glu	Ala	Lys	Ala	Lys	Gln	Arg	Ser	Cys	Gly	Leu	Val	Pro	Arg	Gly	Ser
	225					230					235					240
	Gly	Pro	Gly	Ser	Lys	Ala	Pro	Gly	Ile	Cys	Ile	Asp	Val	Asp	Asn	Glu
					245					250					255	
15	Asp	Leu	Phe	Phe	Ile	Ala	Asp	Lys	Asn	Ser	Phe	Ser	Asp	Asp	Leu	Ser
			260						265					270		
	Lys	Asn	Glu	Arg	Ile	Glu	Tyr	Asn	Thr	Gln	Ser	Asn	Tyr	Ile	Glu	Asn
20			275					280					285			
	Asp	Phe	Pro	Ile	Asn	Glu	Leu	Ile	Leu	Asp	Thr	Asp	Leu	Ile	Ser	Lys
		290					295					300				
25	Ile	Glu	Leu	Pro	Ser	Glu	Asn	Thr	Glu	Ser	Leu	Thr	Asp	Phe	Asn	Val
	305					310					315					320
	Asp	Val	Pro	Val	Tyr	Glu	Lys	Gln	Pro	Ala	Ile	Lys	Lys	Ile	Phe	Thr
					325					330					335	
30	Asp	Glu	Asn	Thr	Ile	Phe	Gln	Tyr	Leu	Tyr	Ser	Gln	Thr	Phe	Pro	Leu
				340					345					350		
	Asp	Ile	Arg	Asp	Ile	Ser	Leu	Thr	Ser	Ser	Phe	Asp	Asp	Ala	Leu	Leu
35			355					360					365			
	Phe	Ser	Asn	Lys	Val	Tyr	Ser	Phe	Phe	Ser	Met	Asp	Tyr	Ile	Lys	Thr
		370					375					380				
40	Ala	Asn	Lys	Val	Val	Glu	Ala	Gly	Leu	Phe	Ala	Gly	Trp	Val	Lys	Gln
	385					390					395					400
	Ile	Val	Asn	Asp	Phe	Val	Ile	Glu	Ala	Asn	Lys	Ser	Asn	Thr	Met	Asp
					405					410					415	
45	Lys	Ile	Ala	Asp	Ile	Ser	Leu	Ile	Val	Pro	Tyr	Ile	Gly	Leu	Ala	Leu
				420					425					430		
	Asn	Val	Gly	Asn	Glu	Thr	Ala	Lys	Gly	Asn	Phe	Glu	Asn	Ala	Phe	Glu
50			435					440					445			
	Ile	Ala	Gly	Ala	Ser	Ile	Leu	Leu	Glu	Phe	Ile	Pro	Glu	Leu	Leu	Ile
		450					455					460				
55	Pro	Val	Val	Gly	Ala	Phe	Leu	Leu	Glu	Ser	Tyr	Ile	Asp	Asn	Lys	Asn
	465					470					475					480
	Lys	Ile	Ile	Lys	Thr	Ile	Asp	Asn	Ala	Leu	Thr	Lys	Arg	Asn	Glu	Lys
					485					490					495	
60	Trp	Ser	Asp	Met	Tyr	Gly	Leu	Ile	Val	Ala	Gln	Trp	Leu	Ser	Thr	Val
				500					505					510		
	Asn	Thr	Gln	Phe	Tyr	Thr	Ile	Lys	Glu	Gly	Met	Tyr	Lys	Ala	Leu	Asn
65			515					520					525			
	Tyr	Gln	Ala	Gln	Ala	Leu	Glu	Glu	Ile	Ile	Lys	Tyr	Arg	Tyr	Asn	Ile

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	530		535		540											
5	Tyr 545	Ser	Glu	Lys	Glu	Lys 550	Ser	Asn	Ile	Asn	Ile 555	Asp	Phe	Asn	Asp	Ile 560
	Asn	Ser	Lys	Leu	Asn 565	Glu	Gly	Ile	Asn	Gln 570	Ala	Ile	Asp	Asn	Ile 575	Asn
10	Asn	Phe	Ile	Asn 580	Gly	Cys	Ser	Val	Ser 585	Tyr	Leu	Met	Lys	Lys 590	Met	Ile
	Pro	Leu	Ala 595	Val	Glu	Lys	Leu	Leu 600	Asp	Phe	Asp	Asn	Thr 605	Leu	Lys	Lys
15	Asn 610	Leu	Leu	Asn	Tyr	Ile	Asp 615	Glu	Asn	Lys	Leu	Tyr 620	Leu	Ile	Gly	Ser
20	Ala 625	Glu	Tyr	Glu	Lys	Ser 630	Lys	Val	Asn	Lys	Tyr 635	Leu	Lys	Thr	Ile	Met 640
	Pro	Phe	Asp	Leu	Ser 645	Ile	Tyr	Thr	Asn	Asp 650	Thr	Ile	Leu	Ile	Glu	Met 655
25	Phe	Asn	Lys	Tyr 660	Asn	Ser	Glu	Ile	Leu 665	Asn	Asn	Ile	Ile	Leu	Asn	Leu
	Arg	Tyr	Lys 675	Asp	Asn	Asn	Leu	Ile 680	Asp	Leu	Ser	Gly	Tyr 685	Gly	Ala	Lys
30	Val 690	Glu	Val	Tyr	Asp	Gly	Val 695	Glu	Leu	Asn	Asp	Lys 700	Asn	Gln	Phe	Lys
35	Leu 705	Thr	Ser	Ser	Ala	Asn 710	Ser	Lys	Ile	Arg	Val 715	Thr	Gln	Asn	Gln	Asn 720
	Ile	Ile	Phe	Asn	Ser 725	Val	Phe	Leu	Asp	Phe 730	Ser	Val	Ser	Phe	Trp 735	Ile
40	Arg	Ile	Pro	Lys 740	Tyr	Lys	Asn	Asp	Gly 745	Ile	Gln	Asn	Tyr	Ile 750	His	Asn
	Glu	Tyr	Thr 755	Ile	Ile	Asn	Cys	Met 760	Lys	Asn	Asn	Ser	Gly 765	Trp	Lys	Ile
45	Ser 770	Ile	Arg	Gly	Asn	Arg	Ile 775	Ile	Trp	Thr	Leu	Ile 780	Asp	Ile	Asn	Gly
50	Lys 785	Thr	Lys	Ser	Val	Phe 790	Phe	Glu	Tyr	Asn	Ile 795	Arg	Glu	Asp	Ile	Ser 800
	Glu	Tyr	Ile	Asn	Arg 805	Trp	Phe	Phe	Val	Thr 810	Ile	Thr	Asn	Asn	Leu 815	Asn
55	Asn	Ala	Lys	Ile 820	Tyr	Ile	Asn	Gly	Lys 825	Leu	Glu	Ser	Asn	Thr 830	Asp	Ile
	Lys	Asp	Ile 835	Arg	Glu	Val	Ile	Ala 840	Asn	Gly	Glu	Ile	Ile	Phe 845	Lys	Leu
60	Asp	Gly 850	Asp	Ile	Asp	Arg	Thr 855	Gln	Phe	Ile	Trp	Met 860	Lys	Tyr	Phe	Ser
	Ile 865	Phe	Asn	Thr	Glu	Leu 870	Ser	Gln	Ser	Asn	Ile 875	Glu	Glu	Arg	Tyr	Lys 880
65	Ile	Gln	Ser	Tyr	Ser 885	Glu	Tyr	Leu	Lys	Asp 890	Phe	Trp	Gly	Asn	Pro 895	Leu

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Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly Asn Lys Asn Ser
 900 905 910
 5 Tyr Ile Lys Leu Lys Lys Asp Ser Pro Val Gly Glu Ile Leu Thr Arg
 915 920 925
 Ser Lys Tyr Asn Gln Asn Ser Lys Tyr Ile Asn Tyr Arg Asp Leu Tyr
 930 935 940
 10 Ile Gly Glu Lys Phe Ile Ile Arg Arg Lys Ser Asn Ser Gln Ser Ile
 945 950 955 960
 Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr Leu Asp Phe Phe
 965 970 975
 15 Asn Leu Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys Tyr Phe Lys Lys
 980 985 990
 20 Glu Glu Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp Ser Asp Glu Phe
 995 1000 1005
 Tyr Asn Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln Pro Thr Tyr Ser
 1010 1015 1020
 25 Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr Asp Glu Ile Gly
 1025 1030 1035 1040
 Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Ile Val Phe Glu Glu
 1045 1050 1055
 30 Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu Lys Glu Val Lys
 1060 1065 1070
 35 Arg Lys Pro Tyr Asn Leu Lys Leu Gly Cys Asn Trp Gln Phe Ile Pro
 1075 1080 1085
 Lys Asp Glu Gly Trp Thr Glu
 1090 1095
 40
 <210> 8
 <211> 1084
 <212> PRT
 45 <213> Artificial Sequence
 <220>
 <223> Description of Artificial Sequence:construct
 50 <400> 8
 Met Leu Ser Arg Ala Val Cys Gly Thr Ser Arg Gln Leu Ala Pro Ala
 1 5 10 15
 55 Leu Gly Tyr Leu Gly Ser Arg Gln Lys His Ser Arg Gly Ser Pro Ala
 20 25 30
 Leu Pro Tyr Pro Tyr Asp Ala Leu Glu Pro His Ile Asp Lys Glu Thr
 35 40 45
 60 Met Asn Ile His His Thr Lys His His Asn Thr Tyr Val Thr Asn Leu
 50 55 60
 Asn Ala Ala Leu Glu Gly His Pro Asp Leu Gln Asn Lys Ser Leu Glu
 65 70 75 80
 65 Glu Leu Leu Ser Asn Leu Glu Ala Leu Pro Glu Ser Ile Arg Thr Ala
 85 90 95

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	Val	Arg	Asn	Asn	Gly	Gly	Gly	His	Ala	Asn	His	Ser	Leu	Phe	Trp	Thr	
				100					105					110			
5	Ile	Leu	Ser	Pro	Asn	Gly	Gly	Gly	Glu	Pro	Thr	Gly	Glu	Leu	Ala	Asp	
			115					120					125				
	Ala	Ile	Asn	Lys	Lys	Phe	Gly	Ser	Phe	Thr	Ala	Phe	Lys	Asp	Glu	Phe	
		130					135					140					
10	Ser	Lys	Ala	Ala	Ala	Gly	Arg	Phe	Gly	Ser	Gly	Trp	Ala	Trp	Leu	Val	
	145					150					155					160	
	Val	Asn	Asn	Gly	Glu	Leu	Glu	Ile	Thr	Ser	Thr	Pro	Asn	Gln	Asp	Ser	
15					165					170					175		
	Pro	Ile	Met	Glu	Gly	Lys	Thr	Pro	Ile	Leu	Gly	Leu	Asp	Val	Trp	Glu	
				180					185					190			
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		210					215					220					
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30					245					250					255		
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				260					265					270			
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			275					280					285				
	Tyr	Arg	Asn	Asn	Leu	Asp	Glu	Val	Ile	Leu	Asp	Tyr	Asn	Ser	Glu	Thr	
		290					295					300					
40	Ile	Pro	Gln	Ile	Ser	Asn	Gln	Thr	Leu	Asn	Thr	Leu	Val	Gln	Asp	Asp	
	305					310					315					320	
	Ser	Tyr	Val	Pro	Arg	Tyr	Asp	Ser	Asn	Gly	Thr	Ser	Glu	Ile	Glu	Glu	
45					325					330					335		
	His	Asn	Val	Val	Asp	Leu	Asn	Val	Phe	Phe	Tyr	Leu	His	Ala	Gln	Lys	
				340					345					350			
50	Val	Pro	Glu	Gly	Glu	Thr	Asn	Ile	Ser	Leu	Thr	Ser	Ser	Ile	Asp	Thr	
			355				360						365				
	Ala	Leu	Ser	Glu	Glu	Ser	Gln	Val	Tyr	Thr	Phe	Phe	Ser	Ser	Glu	Phe	
		370					375					380					
55	Ile	Asn	Thr	Ile	Asn	Lys	Pro	Val	His	Ala	Ala	Leu	Phe	Ile	Ser	Trp	
	385					390					395					400	
	Ile	Asn	Gln	Val	Ile	Arg	Asp	Phe	Thr	Thr	Glu	Ala	Thr	Gln	Lys	Ser	
60					405					410					415		
	Thr	Phe	Asp	Lys	Ile	Ala	Asp	Ile	Ser	Leu	Val	Val	Pro	Tyr	Val	Gly	
				420					425					430			
65	Leu	Ala	Leu	Asn	Ile	Gly	Asn	Glu	Val	Gln	Lys	Glu	Asn	Phe	Lys	Glu	
			435				440						445				
	Ala	Phe	Glu	Leu	Leu	Gly	Ala	Gly	Ile	Leu	Leu	Glu	Phe	Val	Pro	Glu	

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5	Leu 465	Leu	Ile	Pro	Thr	Ile 470	Leu	Val	Phe	Thr	Ile 475	Lys	Ser	Phe	Ile	Gly 480			
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10	Met	Glu	Arg	Glu 500	Thr	Lys	Trp	Lys	Glu 505	Ile	Tyr	Ser	Trp	Ile 510	Val	Ser			
	Asn	Trp	Leu	Thr	Arg	Ile	Asn	Thr 520	Gln	Phe	Asn	Lys	Arg 525	Lys	Glu	Gln			
15	Met	Tyr 530	Gln	Ala	Leu	Gln	Asn 535	Gln	Val	Asp	Ala	Ile 540	Lys	Thr	Val	Ile			
20	Glu 545	Tyr	Lys	Tyr	Asn	Asn 550	Tyr	Thr	Ser	Asp	Glu 555	Arg	Asn	Arg	Leu	Glu 560			
	Ser	Glu	Tyr	Asn	Ile 565	Asn	Asn	Ile	Arg	Glu 570	Glu	Leu	Asn	Lys	Lys 575	Val			
25	Ser	Leu	Ala	Met 580	Glu	Asn	Ile	Glu	Arg 585	Phe	Ile	Thr	Glu	Ser 590	Ser	Ile			
	Phe	Tyr 595	Leu	Met	Lys	Leu	Ile	Asn 600	Glu	Ala	Lys	Val	Ser 605	Lys	Leu	Arg			
30	Glu 610	Tyr	Asp	Glu	Gly	Val	Lys 615	Glu	Tyr	Leu	Leu	Asp 620	Tyr	Ile	Ser	Glu			
35	His 625	Arg	Ser	Ile	Leu	Gly 630	Asn	Ser	Val	Gln	Glu 635	Leu	Asn	Asp	Leu	Val 640			
	Thr	Ser	Thr	Leu	Asn 645	Asn	Ser	Ile	Pro	Phe 650	Glu	Leu	Ser	Ser	Tyr 655	Thr			
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45	Asp 690	Ile	Ser	Gly	Tyr	Gly	Ser 695	Asn	Ile	Ser	Ile	Asn 700	Gly	Asp	Val	Tyr			
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	Ser	Glu	Val	Asn	Ile 725	Ala	Gln	Asn	Asn	Asp 730	Ile	Ile	Tyr	Asn	Gly 735	Arg			
55	Tyr	Gln	Asn	Phe 740	Ser	Ile	Ser	Phe	Trp 745	Val	Arg	Ile	Pro	Lys 750	Tyr	Phe			
	Asn	Lys	Val	Asn	Leu	Asn	Asn	Glu 760	Tyr	Thr	Ile	Ile	Asp 765	Cys	Ile	Arg			
60	Asn 770	Asn	Asn	Ser	Gly	Trp	Lys 775	Ile	Ser	Leu	Asn	Tyr 780	Asn	Lys	Ile	Ile			
65	Trp 785	Thr	Leu	Gln	Asp	Thr 790	Ala	Gly	Asn	Asn	Gln 795	Lys	Leu	Val	Phe	Asn 800			
	Tyr	Thr	Gln	Met	Ile 805	Ser	Ile	Ser	Asp	Tyr 810	Ile	Asn	Lys	Trp	Ile 815	Phe			

	Val Thr Ile	Thr Asn Asn Arg Leu Gly Asn Ser Arg Ile Tyr Ile Asn	820	825	830											
5	Gly Asn Leu	Ile Asp Glu Lys Ser Ile Ser Asn Leu Gly Asp Ile His	835				840					845				
	Val Ser Asp	Asn Ile Leu Phe Lys Ile Val Gly Cys Asn Asp Thr Arg				855					860					
10	Tyr Val Gly	Ile Arg Tyr Phe Lys Val Phe Asp Thr Glu Leu Gly Lys			870					875						880
	Thr Glu Ile	Glu Thr Leu Tyr Ser Asp Glu Pro Asp Pro Ser Ile Leu			885				890						895	
15	Lys Asp Phe	Trp Gly Asn Tyr Leu Leu Tyr Asn Lys Arg Tyr Tyr Leu			900			905					910			
20	Leu Asn Leu	Leu Arg Thr Asp Lys Ser Ile Thr Gln Asn Ser Asn Phe			915			920					925			
	Leu Asn Ile	Asn Gln Gln Arg Gly Val Tyr Gln Lys Pro Asn Ile Phe			930			935					940			
25	Ser Asn Thr	Arg Leu Tyr Thr Gly Val Glu Val Ile Ile Arg Lys Asn			945			950			955					960
	Gly Ser Thr	Asp Ile Ser Asn Thr Asp Asn Phe Val Arg Lys Asn Asp			965				970						975	
30	Leu Ala Tyr	Ile Asn Val Val Asp Arg Asp Val Glu Tyr Arg Leu Tyr			980			985						990		
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	Thr Ser Asn	Ser Asn Asn Ser Leu Gly Gln Ile Ile Val Met Asp Ser			1010			1015				1020				
40	Ile Gly Asn	Asn Cys Thr Met Asn Phe Gln Asn Asn Asn Gly Gly Asn			1025			1030			1035					1040
	Ile Gly Leu	Leu Gly Phe His Ser Asn Asn Leu Val Ala Ser Ser Trp			1045				1050						1055	
45	Tyr Tyr Asn	Asn Ile Arg Lys Asn Thr Ser Ser Asn Gly Cys Phe Trp			1060			1065					1070			
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	<212>	PRT														
	<213>	Artificial Sequence														
60	<220>															
	<223>	Description of Artificial Sequence: polypeptide														
		comprising a mitochondrial leader from human MnSOD														
		and B. Stearothermophilus SOD														
65	<400>	9														
	Met Leu Ser	Arg Ala Val Cys Gly Thr Ser Arg Gln Leu Ala Pro Ala														
	1					5				10					15	

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Leu Gly Tyr Leu Gly Ser Arg Gln Lys His Ser Arg Gly Ser Pro Ala
 20 25 30

5 Leu Pro Tyr Pro Tyr Asp Ala Leu Glu Pro His Ile Asp Lys Glu Thr
 35 40 45

Met Asn Ile His His Thr Lys His His Asn Thr Tyr Val Thr Asn Leu
 50 55 60

10 Asn Ala Ala Leu Glu Gly His Pro Asp Leu Gln Asn Lys Ser Leu Glu
 65 70 75 80

15 Glu Leu Leu Ser Asn Leu Glu Ala Leu Pro Glu Ser Ile Arg Thr Ala
 85 90 95

Val Arg Asn Asn Gly Gly Gly His Ala Asn His Ser Leu Phe Trp Thr
 100 105 110

20 Ile Leu Ser Pro Asn Gly Gly Gly Glu Pro Thr Gly Glu Leu Ala Asp
 115 120 125

Ala Ile Asn Lys Lys Phe Gly Ser Phe Thr Ala Phe Lys Asp Glu Phe
 130 135 140

25 Ser Lys Ala Ala Ala Gly Arg Phe Gly Ser Gly Trp Ala Trp Leu Val
 145 150 155 160

30 Val Asn Asn Gly Glu Leu Glu Ile Thr Ser Thr Pro Asn Gln Asp Ser
 165 170 175

Pro Ile Met Glu Gly Lys Thr Pro Ile Leu Gly Leu Asp Val Trp Glu
 180 185 190

35 His Ala Tyr Tyr Leu Lys Tyr Gln Asn Arg Arg Pro Glu Tyr Ile Ala
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40 Glu Ala Lys Ala Lys
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 <213> Artificial Sequence

50 <220>
 <223> Description of Artificial Sequence: modified human
 mitochondrial leader sequence

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60 Leu Gly Tyr Leu Gly Ser Arg Gln
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65 <210> 11
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- 23 -

<220>

<223> Description of Artificial Sequence: modified human
mitochondrial leader sequence

<400> 11

Met Leu Ser Arg Ala Val Cys Gly Thr Ser Arg Gln Leu Ala Pro Ala
1 5 10 15Leu Gly Tyr Leu Gly Ser Arg Gln
20

INTERNATIONAL SEARCH REPORT

 International Application No
 PCT/GB 99/03699

A. CLASSIFICATION F SUBJECT MATTER
 IPC 7 C12N15/53 C12N15/62 C12N9/02 A61K38/44 A61K48/00
 C07K14/33 A61K39/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FIGUEIREDO D M ET AL: "Delivery of recombinant tetanus -superoxide dismutase proteins to central nervous system neurons by retrograde axonal transport" EXPERIMENTAL NEUROLOGY, US, SAN DIEGO, CA, vol. 145, 1997, pages 546-554, XP002102526 the whole document	1,6,7,9, 10,13,15
Y	FUJII J ET AL: "A defect in the mitochondrial import of mutant Mn-superoxide dismutase produced in Sf21 cells." JOURNAL OF BIOCHEMISTRY, (1998 AUG) 124 (2) 340-6. , XP000867725 page 340 -page 341; figures 1,5 -/-	16-22

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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 "P" document published prior to the international filing date but later than the priority date claimed

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 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
 "&" document member of the same patent family

Date of the actual completion of the international search

28 February 2000

Date of mailing of the international search report

15/03/2000

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Authorized officer

Espen, J

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/GB 99/03699

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOWLER C ET AL: "Characterization of the Bacillus stearothermophilus manganese superoxide dismutase gene and its ability to complement copper/zinc superoxide dismutase deficiency in Saccharomyces cerevisiae" JOURNAL OF BACTERIOLOGY, vol. 172, no. 3, 1990, pages 1539-1546, XP000877200 USA abstract	16-22
X	FRANCIS JW ET AL: "CuZn superoxide dismutase (SOD-1): tetanus toxin fragment C hybrid protein for targeted delivery of SOD-1 to neuronal cells" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 25, 1995, pages 15434-15442, XP002131795 MD US abstract; figures 1,4-8	1,6,7,9, 10,13,15
X	US 5 780 024 A (BROWN ROBERT H ET AL) 14 July 1998 (1998-07-14) the whole document	1,6,7,9, 10,13,15

INTERNATIONAL SEARCH REPORT
information on patent family members

International Application No
PCT/GB 99/03699

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5780024 A	14-07-1998	NONE	